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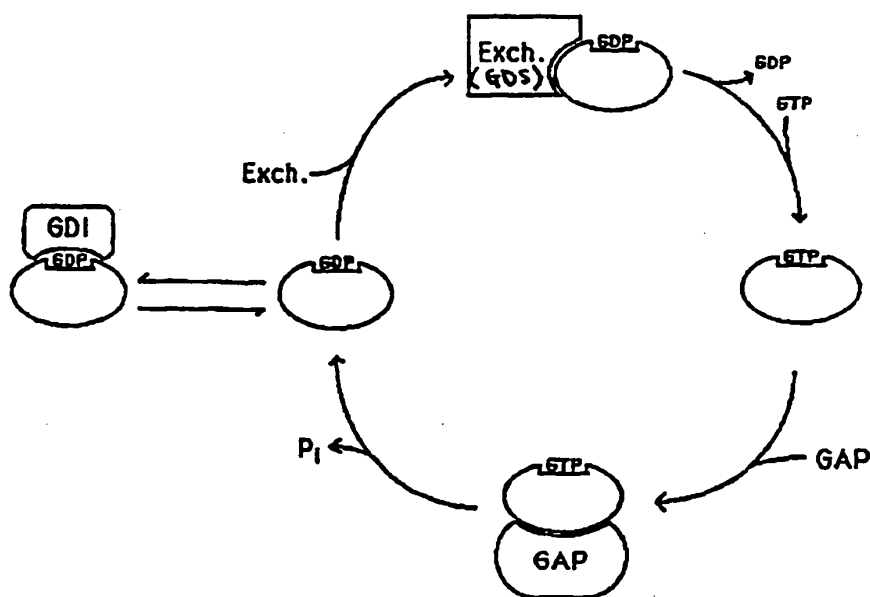
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(21) International Application Number: PCT/US93/12074 (22) International Filing Date: 10 December 1993 (10.12.93) (30) Priority Data: 07/990,337 10 December 1992 (10.12.92) US (71) Applicants: CYTOMED, INC. [US/US]; 840 Memorial Drive, Cambridge, MA 02139 (US). BETH ISRAEL HOSPITAL ASSOCIATION [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US). (72) Inventors: LIM, Bing; 4 Hooper Street, Dorchester, MA 02124 (US). LELIAS, Jean-Michel; 41, rue Louise-de-Karoual, F- 91000 Evry (FR). ADRA, Chaker, N.; 65 East India Row, Apartment 37G, Boston, MA 02110 (US). KO, Jone, L.; 19 Willow Road, Sudbury, MA 01776 (US). (74) Agents: PABST, Patrea, L. et al.; Kilpatrick & Cody, 1100 Peachtree Street, Suite 2800, Atlanta, GA 30309-4530 (US).			(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: **D4 GENE AND METHODS OF USE THEREOF**



Applicants: Mary Cismowski, et al.
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(57) Abstract

The sequence, molecular structure and expression of a cDNA clone, denoted D4, of human and murine origin, preferentially expressed in hematopoietic cells is described herein. The human cDNA clone has been expressed in bacteria and the predicted 24 Kd protein purified. The protein has been used in studies of its biochemical function. As predicted on the basis of sequence, D4 can function as a GDP-dissociation inhibitor of at least several small GTP-binding proteins (CDC42 and rac). The D4 protein was used to generate a polyclonal antibody specific for the protein. The human cDNA was used to obtain several full length murine genomic clones. A clone has been analyzed and sequenced to use for the construction of a gene-targeting vector to produce animals deficient in D4 through disruption of the gene by homologous recombination. These animals can then be used as models for fundamental and applied research on the GTP-binding proteins.

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D4 GENE AND METHODS OF USE THEREOF

Background of the Invention

The United States government has rights in this invention by virtue of support by a National Institute of Health grant No. DK 44099-02.

5 The present invention is generally in the area of regulation of proliferation and differentiation of hematopoietic cells, and is more specifically the nucleic acid sequence encoding a protein called D4, and methods of use thereof in regulation of hematopoietic cells.

10 In the hematopoietic system, a common pluripotent stem cell gives rise to at least eight distinct lineages. While cells of each lineage appear to have strictly defined characteristics and function, a considerable plasticity in lineage specificity at the cellular and molecular level had been observed. For example, leukemic cells had been found to express molecular markers of more than one lineage in the same cell, as reported by Pui CH, Raimondi SC, Head DR, Schell MJ, Rivera GK, Mirro J Jr, Crist WM, Behm FG. (1991) Blood 78:1327-1337; McCulloch EA. (1983) Blood 62:1-13; Bradstock KF, Kirk J, Grimsley PG, Kabral A, Hughes WG. (1989) Br J Haem 75:512; and Greaves MF, Chan LC, Furley AJW, Watt SM, Mulgaard NY. (1986) Blood 67:1-6. It is unclear at the moment whether this reflects a distortion of the genetic mechanism of differentiation (McCulloch) or the normal differentiation process of stem cells (Greaves, et al.). A more dramatic example of this plasticity is the demonstration of the conversion of pre-B cells, which had undergone VDJ rearrangement, into macrophages Klinken SP, Alexander WS, Adams JH. (1988) Cell 53:857-867; and Borzillo GV, Ashmun RA,

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Sherr CJ. (1990) Mol. Cell Biol. 10:2704-2174. These "de-differentiated" cells continue to maintain their immunoglobulin gene rearrangements but morphologically and functionally behave as macrophages.

It is remarkable that cells with a stably rearranged genome can convert into cells of a completely different lineage. These observations suggest that beneath the diversity some or all hematopoietic lineages continue to remain closely related. Hematopoietic cells may then be viewed not as a system of distinctly differentiated cells but as a family of related cells amongst which common features may be detected which distinguish them from other tissues.

It is surmised that there are molecules which regulate molecular events unique to all hematopoietic cells and that these molecules are likely to be important even after commitment into specific lineages.

It is therefore an object of the present invention to provide a gene, and the protein encoded thereby, which is involved in the proliferation and differentiation of hematopoietic cells.

It is a further object of the present invention to provide methods of use for the protein, and inhibition or expression of the gene for the protein, to enhance or limit hematopoietic cell proliferation and differentiation.

Summary of the Invention

By the differential screening of a subtractive hematopoietic-cell cDNA library with subtractive probes, the cDNA clones of several genes not previously identified which are preferentially expressed in hematopoietic cells have been identified

and isolated. The sequence, molecular structure and expression of one of these clones, denoted D4, of human and murine origin, is described herein. Transcripts for D4 are expressed preferentially at a very high level in hematopoietic cells of all lineages. Transcripts are also detected at a lower level in non-hematopoietic cell lines and tissues, including melanoma cells, neuroblastoma cells, skeletal muscles, lung and fat cells. D4 protein expressed from the cDNA in bacteria demonstrated GDP-dissociation inhibitor (GDI)-like activity. D4 is therefore believed to be useful in regulation of proliferation and differentiation of hematopoietic cells.

Using the cDNA, the human D4 gene has been localized to chromosome 12p 12-13, an area involved in chromosomal translocations and deletions of 10% of childhood acute lymphoid leukemia. The cDNA can be used diagnostically in examining DNAs from leukemic patients for identification of sub-types of leukemia. Confirmation of the involvement of D4 in these leukemias will be of great clinical and therapeutic significance. D4 protein will be used as a labelled probe to search for and isolate interacting protein(s).

The human cDNA clone has been expressed in bacteria and the predicted 24 Kd protein purified. The protein has been used in studies of its biochemical function. As predicted on the basis of sequence, D4 can function as a GDP-dissociation inhibitor of at least several small GTP-binding proteins (CDC42 and rac).

The D4 protein was used to generate a polyclonal antibody specific for the protein. The antibody can be used to identify other putative small GTP-binding proteins which interact with D4. The antibody will also be useful in diagnostic and research

applications.

The human cDNA was used to obtain several full length murine genomic clones. A clone has been analyzed and sequenced to use for the construction of a gene-targeting vector to produce animals deficient in D4 through disruption of the gene by homologous recombination. These animals can then be used as models for fundamental and applied research on the GTP-binding proteins.

It is believed that D4 activity is required for progression of differentiation and growth; a reduced or absence of D4 activity would arrest terminal differentiation. This thesis is supported by the following observations: a) a dramatic increase in D4 transcripts during proliferation and differentiation of the earliest hematopoietic precursors isolated from day 9 murine yolk sac cells and b) different changes in the level of D4 transcripts during induced differentiation of various hematopoietic cell lines. It is also supported by studies that show that the D4 protein is sometimes present in the cytoplasm, sometimes in the nucleus, and sometimes in both, indicating that in some cells the protein is involved in nuclear events. Additional evidence is presented that D4 functions as a GDI for small ras-like GTP-binding proteins. D4 also appears to have activity as a suppressor of transformation, by functioning as a negative regulator of activation of ras related GTP-binding proteins through its GDI activity, as demonstrated by comparative studies with p53, a known tumor suppressor.

Therefore, potential clinical use of D4 involves the manipulation of cell growth and differentiation in normal cells and in treatment of some leukemias or potentially other types of tumors. For example, to induce differentiation of leukemic cells defective in the gene, D4 would be administered to cells using

protein carriers to effectively deliver the protein into cells. To transiently arrest differentiation such as during efforts to amplify hematopoietic stem cells *in vitro* before bone-marrow transplantation, the production of D4 protein would be inhibited using anti-sense oligonucleotides or ribozyme technology.

Comparison between D4 and rhoGDI, and analysis of the functional activity of rhoGDI and D4 chimeric proteins and truncated proteins, indicates that the C-terminal region (within the last 33 amino acids of rhoGDI) of both proteins confers substrate specificity, in the case of rhoGDI for rho protein, and in the case of D4, for an unknown protein which can be isolated based on this knowledge.

Brief Description of the Drawings

Figures 1A and 1B are a Northern analysis of D4 mRNA in human hematopoietic and non-hematopoietic cell lines. Figure 2A is 15 μ g of total RNA resolved on a denaturing agarose gel, transferred to Hybond-NTM filters (Amersham), hybridized with ³²P-labelled D4 cDNA probe and washed with 0.2 x SSC at 65°C before autoradiography for 24 hrs at -80°C. Figure 2B is the gel of Figure 2A stained with Ethidium bromide to monitor integrity of samples and constancy of loading. Tissues are hemapoietic cells: K562, OCIR, KG-1, LY17, OCI-MYI, DU528, and bone marrow; and non-hematopoietic cells: BS-1, HEPG2, CALU-1, SKMEL, HS294, HELA, HUSK, and SKNSH cells.

Figures 2A and 2B are a Northern analysis of D4 in 15 μ g RNA samples extracted from normal human tissues. Figure 2A is an autoradiograph of the gel. Figure 2B is the gel of Figure 2A stained with Ethidium Bromide. Tissues are bone marrow, T-lymphocyte, skin fibroblast, brain, kidney, lung, liver, adrenal and skeletal muscle.

Figures 3A and 3B are a Northern analysis of D4 in RNAs (15 μ g/lane) extracted from normal murine tissues. Figure 3A is the hybridized membrane was washed at 0.2 X SSC, 65°C for 1 hr and autoradiographed for 24 hrs at -80°C. Lower stringency hybridizing and washing condition did not reveal additional or stronger signals. Figure 3B is gel stained with Ethidium Bromide.

Figures 4A, 4B, 4C, 4D, and 4E is a Northern analysis of (A) D4, (B) embryonic globin β h1, (C) murine macrophage mannose receptor MØMR and (D) MAC-1 in RNAs of ES colonies pooled and collected after different days in culture for hematopoietic differentiation. The same blot was probed sequentially A-D. (E) is an ethidium bromide picture of the gel.

Figure 5 is a Northern analysis of hematopoietic cell lines at different times after initiation of induction, control, 0.5, 2, 8, 24, and 48 hr. Figure 5A is U937 induced with TPA. The same blot was probed sequentially, (A) with D4 and (S) with CD11b. Figure 5B is HL60 induced with DMSO. Figure 5C is MEL induced with DMSO.

Figure 6 is a comparison of murine (m) (Sequence ID No. 6) and human (h) (Sequence ID No. 4) D4 with bovine (b) (Sequence ID No. 8) and human (h) rhoGDI protein sequences using the FASTA program of Pearson and Lipman, (1988) Proc. Natl. Acad. Sci. 85:2444-2448. Vertical lines indicate identical matches and asterisks indicate conservative changes. Gaps introduced to maximize alignment are indicated by dots.

Figures 7A and 7B are a Northern analysis of D4 mRNA in total RNA from day 9 murine yolk-sac cells and hematopoietic cells developing in colonies derived from precursor cells after different days in *in vitro* cultures.

Figure 8 is a diagram of the vector and scheme used to generate the D4 (D4)-fusion protein using the polylinker of Sequence ID No. 7.

Figures 9A and 9B are SDS PAGE profiles of purification steps for the malE-D4 fusion protein expressed in *E. coli*. Figure 9A is the purification of malE-D4 from *E. coli*. The proteins were stained with Coomassie brilliant blue. Lanes: 1, protein molecular weight marker; 2, bacterial homogenate before IPTG induction; 3, bacterial homogenate after IPTG induction; 4, bacterial pellet after sonication; 6, eluted pools from amylose column; 7, eluted pools from Q-SepharoseTM column; 7, molecular weight markers. Figure 9B is the cleavage of D4 from malE-D4 with factor Xa and further purification for iD4. 10 μ g factor X was added to maltose free mal-D4 protein solution and digestion completed after 12 hours incubation at room temperature. Male was separated from D4 by passing through amylose column. Male binds to amylose and D4, approximately 90% pure, was collected in flow through fractions. Lanes 1 and 2 eluted pools from Q-SepharoseTM column; lane 3, pure D4.

Figures 10A, 10B and 10C are assays for GDI activity of D4. Figure 10A is the principle of the GDI assay. Figure 10B is the dose response of the rhoGDI GDI activity on CDC42. The *S. frugiperda*-expressed CDC42Hs was pre-incubated with 7 μ M [α -³²P]GTP for 25 min at room temperature. This incubation converts all of the bound GTP to GDP. The [α -³²P]GTP-labeled CDC42Hs proteins (approximately 15 ng) were then incubated with the indicated amounts of Mono-S-purified CDC42Hs-GDI activity in the presence of 2.5 mM EDTA and no added MgCl₂. After 6 minutes, the samples were filtered on nitrocellulose (BA85) filters and the amount of the [α -³²P]GTP which remained bound to the CDC42Hs protein relative to the

amount of radiolabelled guanine nucleotide that was bound at the start of the assay, as a function of the amount of GDI added to the assay incubation, was determined. Figure 10C is the dose response of D4-GDI activity on CDC42Hs. Figure 10D is the dose response of D4 -GDI activity on rac 1.

Figure 11 is a graph of the dose response profiles of GDI activity for chimeric proteins, GST-GDI (circles); GST-L_NG_C (triangles); GST-mD4 (dark squares); GST-LD4 (open squares); and GST-G_NL_C (diamonds), measured as percent GDI activity versus concentration of fusion protein (μ M).

Figures 12A, 12B, 12C, and 12D are graph showing suppression of rat fibroblast transformation by D4, measured as foci counts at day 14-18 after transfection, which represent total transformed colonies obtained from one transfection. In Figures 12A, 12B, 12C and 12D, 3 μ g oncogene DNA (ras, E1A or p53m) was used. In Figures 12A, 12B, and 12C, 9 μ g of p53wt (tumor suppressor) or D4 was used. In 12D, 3 μ g, 9 μ g, or 12 μ g of either CMVv (control), p53wt, or CMV-D4 was added. Figure 12A compares the effect of ras, E1A (shaded); ras, E1A, p53wt (hatched); and ras, E1A, and D4 (either with the promoter pgk or CMV; dark). Figure 12B compares the effect of E1A, p53m (hatched); E1A, p53m, p53wt (hatched); and E1A, p53m, CMV-D4 (dark). Figure 12C compares the effect of ras, p53m (shaded); ras, p53m, p53wt (hatched); and ras, p53m, D4 (either with the promoter pgk or CMV; dark). Figure 12D compares the effect of ras, E1A, and one of CMVv, p53wt, or CMV-D4 by dosage of the latter.

Figure 13 is a schematic depiction of GDI in inhibition of GDP dissociation from the GTP-binding protein, 'G'. An active GTP-bound form is generated from the inactive GDP-bound form by nucleotide exchange catalyzed by GDS, the GDP-dissociation

stimulator. The GTPase activating protein, GAP, increases the inherent GTPase activity of the 'G' protein by several hundred fold, and converts the active GTP-bound form into the inactive GDP-bound form again through hydrolysis of the GTP.

Detailed Description of the Invention

I. Isolation, Cloning and Sequencing of a gene encoding D4.

A gene which has not previously been reported was isolated from a clone predominantly expressed in hematopoietic cells. The D4 cDNA encodes a protein with homology to bovine rhoGDI, recently described by Fukumoto, et al., (1990), a GDP-dissociation inhibitor (GDI) for the rho(ras-homologous) proteins (although homologous, the sequence and structural homology is insufficient to use probes from the rhoGDI to obtain the D4 gene).

Human and murine D4 cDNA and the gene were isolated as described below. The equivalent gene could be isolated from other mammalian species using the same methodology.

MATERIALS AND METHODS

Cell Lines. Hematopoietic cell-lines included pluripotential (DU528, K562), erythro-leukemia (HEL, OCIR), monoblastic (U937), myeloblastic (KG-1), promyelocytic (HL60), T-cell lymphoma and leukemia (LY-17, Molt-4), myeloma (OCIMy1), megakaryocytic (Dami) and murine erythroleukemia (MEL) cells. Non-hematopoietic cell-lines were bone marrow stromal (BS-1), hepatoma (HepG2), lung cancer (Calu-1), cervical cancer (HeLa), melanoma (HS294, SKMEL), skeletal muscle (HuSk), neuroblastoma (SKNSH), and skin fibroblasts. All cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and

1mM L-glutamine, except for DU528 where horse serum replaced FBS. All cell lines were obtained from the American Type Culture Collection (ATCC) except where acknowledged.

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Construction of subtractive cDNA libraries.

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Three hematopoietic cell lines (K562, KG-1 and DU528) and a non hematopoietic human bone-marrow stromal line (BS-1) were used in the construction of subtractive cDNA libraries and generation of probes for differential screening. cDNAs made from the four cell lines were hybridized with an excess of BS-1 mRNA. Unhybridized cDNAs were separated by a cDNA-mRNA hybridization technique as described by Sive et al (1988) Nucleic Acid Research 16:10937. mRNAs of BS-1 were biotinylated with photobiotin acetate (Invitrogen) following protocol recommended by vendor. cDNA biotinylated mRNA hybrids and excess biotinylated mRNAs were removed by treatment with streptavidin (Invitrogen) followed by phenol extraction leaving unhybridized cDNAs in the aqueous phase. Two rounds of subtractive hybridization were performed. The resulting unhybridized cDNAs were used to construct four libraries (K562/BS-1, KG-1/BS-1, DU528/BS-1 and BS-1/BS-1) as described by Caput et al (1986) PNAS 83:1670-1674, using the PT3T719U multiphagemid vector (Pharmacia). cDNAs were cloned directionally into the Pst I and BamHI sites.

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Generation of DNA probes from subtractive libraries. Recombinant DNA was purified from the KG-1/BS-1, DU528/BS-1 and BS-1/BS-1 subtractive libraries. cDNA inserts were released by restriction enzymes PstI and BamHI, purified on low-melt agarose electrophoretic gel and labelled with 12p using multi-prime labelling (Pharmacia).

Cloning of murine D4. A phage cDNA library in lambdaASHlox vector (Novagen, Madison, WI) derived

from 15 day embryo RNAs was screened with the human D4 cDNA following the technique described by (Sambrook et al (1989) Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory Press). Hybridization and washing was carried out under high stringency conditions. Positive clones were identified by radiography and individual clones isolated by two rounds of secondary screening. DNA from selected clones were extracted as described. cDNA was analyzed by Southern analysis after restriction digestion.

In vitro differentiation of murine Embryonal Stem (ES) cells. The CCE ES line was maintained and passaged as described by Robertson EJ. (1987) Teratocarcinomas and embryonic stem cells, a practical approach. EJ Robertson, Ed. 71-112 (IRL Press) in the presence of Leukemic Inhibitory Factor (LIF, Genetics Institute, Cambridge). Differentiation of totipotent ES cells into hematopoietic cells was studied with an *in vitro* assay similar to that described by Wiles et al., (1991) Development 111:259-267. Single cell suspension of the CCE cell line were cultured in non-tissue culture 35 mm dishes (Fisher) with a mixture containing 0.9% methyl-cellulose (Fluka), 20% FBS (Hyclone), 1% bovine serum albumin, 2 u/ml erythropoietin (Dr.D Worchowski, Pennsylvania State University), 50 ng/ml Stem cell factor (Amgen) and 200-400 u/ml IL-1 (Hoffman LaRoche) at a cell concentration of 1000-2000 cells per ml. Incubation was carried out in a humidified atmosphere at 37°C and colonies examined and collected at different days after initiation of culture by pooling colonies from several dishes.

Induction of cell lines. The cell lines U937, HL60 and MEL were grown to a concentration of 5×10^5 cells/ml before initiation of induction: U937 was

induced with $5 \times 10^{-8} \text{ M}$ 12-O-tetradecanoyl-phorbol-13-acetate (TPA, Sigma); HL60 was induced with TPA or 4% dimethyl sulfoxide (DMSO); MEL was induced with 1.5% DMSO.

5 **General Methods.** Standard methods were carried out as described in Sambrook et al (1989) Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory Press, the teachings of which are specifically incorporated herein. RNA was isolated
10 using the guanidinium isothiocyanate/CsCl procedure. DNA sequencing was done by the dideoxynucleotide chain-termination technique of Sanger F, Milken S, Coulson AR (1977) Proc. Natl. Acad. Sci. 74:5463-5467 after subcloning appropriate DNA
15 fragments into M13.

Chromosomal mapping. D4 gene was localized by the fluorescent in-situ hybridization (FISH) technique of Pinkle, et al. (1988) Proc. Natl. Acad. Sci. 85:9138-9142. Metaphase chromosomes were obtained
20 from phytohemagglutinin (PHA) stimulated peripheral blood cells. Fluorescence-conjugated human D4 cDNA was hybridized to chromosomal spreads and processed as described by Weier HU, Zitzelsberger, Gray JW. (1991) Bio Techniques. More than twenty metaphases
25 were examined and analyzed under UV-light activated microscopy.

RESULTS

Construction of subtractive libraries. cDNA libraries of hematopoietic cell lines that are
30 enriched for cDNAs of mRNA preferentially or uniquely expressed in hematopoietic cells were constructed. This was achieved by subtracting cDNAs of the hematopoietic cell-lines with mRNAs from a non-hematopoietic cell-line. The hematopoietic cell
35 lines were chosen on the basis of their primitive stem cell characteristics. K562 cells express markers for erythroid, granulocytic and

megakaryocytic lineages, as reported by McCulloch EA. (1983) Blood 62:1-13. KG-1 cells, a myeloblastic line, express the CD34 surface antigen, as reported by Satterthwaite AB, et al., (1990) Blood 75:2299-2304, a marker for multipotential stem cells. DU528 cells, derived from a patient with leukemia, have been shown by Kurtzberg, et al. (1985) J. Exp. Med. 162:1561-1578, to be capable of differentiating into both lymphoid and myeloid lineages. Since the objective of subtraction was to reduce the complexity of the libraries, the choice of the non-hematopoietic mRNAs used for subtraction was made arbitrarily, a human bone-marrow stromal derived line, BS-1, described by Scadden, et al., (1990) Blood 76:317-322. Two rounds of subtraction were performed before the cDNAs were used to construct the libraries. A "normalized" stromal BS-1 cDNA library enriched for low frequency molecular species was also constructed using the stromal cDNAs obtained after subtracting twice with stromal mRNAs.

Differential screening and identification of hematopoietic specific clones. The established libraries became a ready source of probes. cDNA inserts from two hematopoietic (DU528 and KG-1) and the BS-1 subtractive libraries were released and purified. The three probes were then used to hybridize against triplicates of the K562 library. Colonies hybridizing with the hematopoietic-probes (DU528 and KG-1) and not with the BS-1 probe were isolated and expanded. DNAs from these clones were extracted and used as individual probes to examine Northern blots consisting of a panel of total RNAs from hematopoietic and non-hematopoietic cell lines. The patterns of expression allowed us to identify cDNA clones of different mRNAs which are either preferentially or specifically expressed in hematopoietic cells.

Identification of clone D4. Clone D4 contains an insert which detects a 1.5 kb. D4 transcript is present in great abundance in hematopoietic cell lines of myeloid and lymphoid, as shown by Figure 1. In contrast, non-hematopoietic cell lines either do not express the transcript (HepG2, HeLa) or do so at a much lower level. Only cell lines of bone-marrow stroma (BS-1) and lung (Calu-1) expressed a comparable level of D4 but even in these cells, the levels of D4 expressed were consistently lower than in hematopoietic cells.

Expression of D4 in normal human and murine tissues. Total RNAs from various normal human tissues was examined to compare with the results from cell lines, as shown by Figures 2A and 2B. Human RNAs, except bone marrow, T-lymphocytes and skin fibroblasts, was a kind gift of Dr. B. Seizinger (Massachusetts General Hospital, Boston). Bone marrow cells and peripheral blood T lymphocytes expressed a high level of the transcript. In contrast a weaker signal was seen in the lung while a significantly lower level was seen in other non-hematopoietic tissues, including kidney, liver, adrenal and muscle. A barely detectable signal was seen in brain cells and no transcript was detected in skin fibroblasts. Examination of RNAs from murine tissues with the human CDNA also detected a strongly hybridizing 1.5 kb transcript expressed almost exclusively in hematopoietic tissues, as shown by Figures 3A and 3B. These included bone marrow cells, spleen, thymus and lymph nodes.

Molecular characterization of human D4. Sequence ID No. 3 is the nucleotide sequence and Sequence ID No. 4 is the deduced amino acid sequence of clone D4. The amino acid sequence of the longest open reading frame is shown numbered from the presumed initiating methionine. The TGA stop codon

(END) is followed by a 3' untranslated region containing an AATAAA poly(A) addition sequence (underlined). The in-frame upstream stop codon, TAG, is at position 66. The consensus N-glycosylation sites (N-X-S/T) are at nucleotides 594-602 and 648-656.

The cDNA contains an putative open reading frame encoding a protein of 201 amino acids with a calculated molecular weight of 23 kd. This is consistent with the preliminary observation of a 23 kd to 24 kd protein obtained by expression of the cDNA in bacteria. Analysis with the Kyte-Doolittle algorithm (Kyte J, Doolittle RF. (1982) J. Mol. Biol. 157:105-132) revealed the protein to be markedly hydrophilic in its overall characteristic with no hydrophobic domain indicative of a membrane spanning region. A search in the Genbank data base, using the FASTA program ((Pearson WR, Lipman DJ (1988) Proc. Natl. Acad. Sci. 85:2444-2448), revealed that clone D4 bears some homology to the bovine rhoGDI cDNA (Fukumoto, et al. (1990) Oncogene 5:1321-1328). They share a 60% identity at the nucleic acid level while the predicted protein sequences are 67% identical. The homology is distributed throughout the cDNA. The greatest divergence is seen within the first 25 amino acids where identical residues dropped to 16%. No significant homology to other genes, particularly other regulators of G-proteins, was identified.

Expression of D4 during differentiation of ES cells. The regulation of the D4 gene expression during the transition of uncommitted embryonic cells into hematopoietic cells was studied to examine the expression of D4 during the earliest events of hematopoiesis. An in vitro system (Wiles MV, Keller G. (1991) Development 111:259-267; Schmitt RM, Bruyns E, Snodgrass HR. (1991) Genes & Development

5:728-740) whereby murine ES cells, cultured in semi-solid culture conditions, undergo development from single cells into large colonies containing hematopoietic cells of the erythroid, granulocytic and megakaryocytic lineages. Colonies at different days after culture were harvested and total RNA extracted. In a typical experiment, colonies at day 9-10 will display hematopoiesis most easily recognized under inverted light microscopy by a dense ring of erythroid cells mixed with non-erythroid cells or a central core of blood island.

Figure 4A, 4B, 4C, 4D, and 4E are Northern blots of a panel of RNAs from colonies at days 2, 4, 6, 8, 10 and 12. The filter was probed for D4 and mRNA of several other genes indicative of development along different hematopoietic lineages; embryonic globin β h1 for primitive erythropoiesis (Whitelaw E, Tsai SF, Hogben P, Orkin SH. (1990) Mol. Cell. Biol. 10:6596-6606), Mac-I (an integrin cell surface antigen) for mature granulocytes and macrophages (Miller LJ, Schwarting R, Springer TA (1986) J. Immunol. 137:2891-2895) and the macrophage mannose-receptor MØMR, specific for macrophage lineage (Stahl PD (1990) Am. J. Respir. Cell Mol. Biol. 2:317-318). At day 6, before erythroid cells were recognizable in the colonies, transcripts for β h1 globin were clearly detected. MØMR and D4 transcripts were first detectable between day 6 and 8. Mac-I transcripts were first detected at day 10

that different lineages are modulated differently as a result of induction. In U937, a myelomonocytic cell line, the induction of differentiation into macrophages by TPA caused a down regulation of D4 so that by 48 hours only a very low level of D4 was detected (Figure 5A). This was in contrast with the activation and up-regulation of CD11B, the human homologue of Mac-1. When HL60 cells were induced to differentiate into neutrophils (monitored by CD11b expression), no noticeable change in the level of D4 (Figure 5B) was seen. However, when HL60 cell were induced to differentiate into macrophages using TPA, a down regulation of the transcript was observed as in U937 cells. When the murine erythroleukemia cell line, MEL, was induced to undergo terminal differentiation by DMSO, a strong and rapid up-regulation of D4 was evident within the first 2 hours (Figure 5C). Differentiation of the MEL cells were indicated by over 80% benzidine positive cells and the appearance of adult, β -globin transcripts.

Isolating murine D4 CDNA. A total of one million plaques were screened with the human D4 CDNA probe and 11 positive clones were identified. A second round of screening was carried out to isolate 10 individual positive clones. A clone with the longest cDNA insert was selected for sequencing.

Molecular Analysis of murine D4 CDNA. Sequence ID No. 5 is the nucleotide sequence and Sequence ID No. 6 is the predicted amino acid sequence of the murine D4 1107 bp cDNA clone isolated by the human D4 clone. Computer analysis shows that at the nucleotide level, the human and murine cDNAs are about 82% identical. The cDNA contains an open reading frame encoding a protein with 200 amino acids.

Figure 6 shows a comparison of the murine and human D4 protein with the bovine rhoGDI (Fukomoto, et

al., 1990). There is a high degree of conservation from mouse to man, the proteins being almost 90% identical with the similarities evenly distributed through the protein. As a result, one could obtain with routine efforts the same gene from any other mammalian source. This contrasts with a much lower similarity (67%) between human D4 and bovine rhoGDI. As in the human D4, the greatest divergence between murine D4 and rhoGDI is in the first 20 amino acids.

Expression of D4 in differentiating early hematopoietic cells. During murine embryogenesis, the earliest site of hematopoietic activity is in day 7 extra-embryonic yolk sac. At day 9 it is possible to dissect away the yolk sac from the embryo and isolate the cellular contents. Figure 7 demonstrates that the expression of D4 is easily detected in Yolk Sac cells (Y.S. cells) at day 9 gestation. In vitro cultures of this population gave rise to colonies which were collected at days 2.5, 4 and 6. Colony size increased rapidly from 50-100 cells per colony at day 2.5 to about 1000 cells per colony by day 6. At this stage the colonies were a mixture of erythroid, non-erythroid and mixed colonies, similar to what was described by Wong et al (1986) Proc. Natl. Acad. Sci. 83:3851-3854, then the colonies stopped expanding and began to degenerate. There was a tremendous increase in the expression of D4 within 2.5 days of culture. This heightened activity appeared to be maintained throughout further development of the progenies within the colonies.

Chromosomal mapping. The mapping of D4 using the FISH technique shows that it localized to chromosome 12p. A careful analysis of the region revealed that D4 localizes to 12p 12-13.

Examination of both cell lines and normal tissues showed that D4 is expressed at a high level only in hematopoietic cells and is highly conserved

across species. This suggests that D4 has some unique functional significance in hematopoietic cells.

Supportive evidence was obtained by examining the expression of D4 during early hematopoiesis in murine ES cells and during differentiation in inducible hematopoietic cell-lines. ES cells are totipotent embryonal cells capable of developing into every tissue type of the animal (Bradley et al., (1984) Nature 309:255-256). The CCE cell line was maintained in liquid cultures as undifferentiated cells in the presence of LIF. Using the *in vitro* methyl-cellulose culture system described, individual cells developed into mixed-colonies containing erythrocytes, granulocytes and megakaryocytes. The expression of globin genes in these colonies mimic the temporal pattern of embryonic, fetal and adult globin-gene activation in developing embryos (Wiles, et al., (1991); Lindenbaum MH, Grosveld F. (1990) Genes & Development 4:2075-2085). Colonies from such *in vitro* cultures of ES cells, containing targeted disruption of the GATA-1 gene, displayed an abrogation of erythroid cell development similar to what was observed in transgenic animals generated by the same mutated ES cells (Simon, et al., (1992) Nature Genetics 1:92-98). Therefore the assay provides a useful alternative to *in vivo* studies of the expression and function of specific genes during hematopoietic cell development.

The kinetics of D4 expression in these colonies was compared to the expression of three other hematopoietic specific genes, β h1, MØMR and Mac-I, as markers of embryonic erythroid, macrophage and mature granulocytic differentiation. β h1 transcripts were detected the earliest, followed by MØMr and D4. Mac-I was detected at later stage colonies, reflecting the emergence of mature neutrophils and

macrophages in the colonies. Therefore it appears that as early hematopoietic cells develop from embryonic cells, D4 is activated. The difference between embryonic hematopoiesis and definitive adult hematopoiesis is still unclear. That D4 transcripts were seen after the initiation of embryonal globin transcription suggests that D4 might not be relevant during embryonic hematopoiesis or at least not during embryonic erythropoiesis.

D4 expression in a population of primitive progenitors was determined to provide further evidence of the significance of D4 in early hematopoiesis. The first site of hematopoietic activity is in day 7 yolk sac where the cell population consist largely of primitive erythroblasts. A second wave of stem cells and progenitors develops which migrate to the fetal liver to initiate hepatic hematopoiesis. By day 9.5 to 10.5 the liver contains stem cells capable of reconstituting adult hematopoiesis. The cells in day 9 yolk sac can be physically isolated. Wong et al has demonstrated a high frequency of single- and multi-lineage precursors within this population. In contrast, cells from the embryo proper at day 9 do not harbor colony-forming cells. Lui et al has also shown that the day 9 yolk sac cells contain T cell precursors that could reconstitute fetal thymus. Such results indicate that the yolk sac is the source of stem cells that migrate to seed the adult hematopoietic tissues. D4 transcripts were easily detected in day 9 yolk sac cells. Furthermore, when hematopoietic progenitors cells from the yolk sac were allowed to differentiate in vitro into colonies, a dramatic increase in the level of D4 expression was seen. These results together indicate that the D4 prot in is essential during the earliest phases of hemat poietic cell d velopment.

Hematopoietic cell lines induced to undergo differentiation revealed markedly different regulation of D4 expression. During differentiation into macrophages, D4 transcripts became significantly down regulated. In contrast, differentiation into neutrophils showed no obvious change in the level of D4 expressed while differentiation along the erythroid lineage caused a very early transient increase followed by a fall to base-line level.

Taken together, these results suggest that D4 is involved in the regulatory processes of adult hematopoiesis and that it may function differently in different lineages.

II. Expression of D4 Protein.

Generation of fusion proteins of D4.

Construction of expression vector

A D4 expression vector was constructed using the pMAL vector (New England BioLab). A gene segment containing the entire coding region for human D4 protein was generated by the polymerase chain reaction (PCR) technique, using a pair of primers, D4-N2 and D4-C2. D4-N2 (5'-ATT ATG ACT GAA AAA GCCCCA GAG CCACATGTG-3') (Sequence ID No. 1) is a 33 mer spanning from nucleotides 75 to 104 of the D4 sequence and including three additional nucleotides, ATT, located immediately 5' of the ATG site of the D4 gene. D4-C2 (5'-GACTCTAGATCATTCTGTCCACTCCTTCTT-3') (Sequence ID No. 2) is a 30 mer spanning from nucleotides 660 to 680 and containing a newly added XbaI restriction site 3' to the TGA (nucleotides 678-680). The resulting D4 fragment has a length of 618 base pairs.

The D4 DNA segment was digested with the XbaI enzyme and subcloned into the pMAL-C vector (New England Bi Lab, Beverly, MA) which was digested at the StuI and XbaI sites, as shown in Figure 8. The resulting D4 expression vector, pMAL-D4, was used for

the expression of fusion protein, Mal-D4, with a maltose binding domain at its N-terminal half and a complete D4 protein at its C terminal half.

Expression and isolation of D4 protein.

5 The pMLA-D4 vector was transformed into DH5 α bacterial host for the expression of Mal-D4 fusion. Ten ml of an overnight culture of recipient DH5 α cells were seeded into one liter of LB broth containing 100 μ g/ml of ampicillin. The seeded
10 bacterial culture was incubated at 37°C under continuous shaking conditions until it reached 0.45 OD₆₅₀. The induction of the production of the Mal-D4 fusion protein was initiated by the addition of IPTG to a final concentration of 0.3 mM. The culture was
15 allowed to continue for another four hours.

 The induced culture was pelleted and resuspended in 50 mL of lysis buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM sodium azide) containing 1 mg/mL of lysozyme. This cell suspension was
20 subjected to one cycle of freeze-thaw and then sonicated to complete lysis. The lysate was spun at 10,000 g, 40°C for 30 min, and the soluble lysate was collected, diluted five fold with lysis buffer and passed through a 20 mL amylose column (New England Biolab, Beverly, MA). The column was subsequently
25 washed with 100 mL of lysis buffer and eluted with lysis buffer containing 10 mM maltose. Fractions containing mal-D4 protein were pooled, diluted four fold with 20 mM Tris-HCl pH 8.0, passed through 1 mL of Q-sepharoseTM column (Pharmacia, NJ). The Q--sepharoseTM column was then washed with 20 mL of 20 mM
30 Tris-HCl, pH 8.0 with 50 mM NaCl and eluted with buffer containing 20 mM Tris HCl, pH 8.0 and 0.75 M NaCl. The Q-sepharose columnTM was used to remove the free maltose from the mal-D4 fusion protein, prior to
35 cleaving the fusion protein with factor Xa (N w England Biolab, Beverly, MA). 10 μ g of factor Xa was

added to the mal-D4 fusion protein; the digestion was completed after a 12 h incubation at room temperature. To separate D4 protein from the maltose binding domain (mal), the digestion mixture was diluted two fold with lysis buffer and passed through a 20 mL amylose column. While the mal protein bound to the amylose column, the D4 protein was present in the flow-through fractions. In general, the purity of the D4 protein was around 90%. This D4 protein, iD4, is preceded by an extra isoleucine as a result of factor Xa cleavage.

Generation of human D4 fusion protein. Figures 9A and 9B show the purification of a fusion protein of the predicted molecular weight of 71 Kd produced in bacteria. The purification of the fusion protein was followed by the cleavage of the mal-D4 protein with thrombin. Final separation of the pure iD4 protein was achieved by passage through an amylose column giving rise to the expected iD4 protein of molecular weight 24 kd.

III. Biochemical Function studies.

The ability of the iD4 or D4 protein to inhibit the dissociation of GDP from CDC42Hs was measured by nitrocellulose filter binding assay. Specifically, 15 ng of CDC42Hs purified from human platelets as described by Hart et al. (1991) Nature 354:311-314 was incubated with 0.625 μ M GTP- α - 32 P in 20 mM Tris-HCl pH 8.0, 0.725 mM dithiothreitol, 10 mM MgCl₂, 50 mM NaCl, 100 μ M AMP-PNP for a period of 25 minutes. Previous experiments have shown that the GTP binds to CDC42Hs and is completely hydrolyzed to GDP during this incubation. Aliquots of this binding mixture (1/16 of the total) were then diluted 10-fold into a tube containing the indicated amounts of the iD4 fusion protein and a final concentration of 34 mM Tris-HCl pH 8.0, 160 μ M dithiothreitol, 205 mM NaCl, 1 mM MgCl₂, 3.3 mM EDTA, 200 μ M GDP, 220 μ M AMP-PNP,

and 100 μ g/ml bovine serum albumin. After a six minute incubation to allow dissociation, 0.5 ml of cold dilution buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$) was added to the mixture and subsequently filtered on nitrocellulose (BA-85, Schleicher and Schuell). The filters were washed 3 times with dilution buffer to remove free nucleotide, and the radioactivity associated with the filter was determined by liquid scintillation counting.

Assay for GDI and GDS activity of D4. The murine cDNA for D4 and the predicted protein is 89% identical to the human protein. The data presented here shows that the murine D4 is almost 90% identical to the human protein. D4 and rhoGDI are 67% homologous and are therefore most likely members of a family of GTP-binding protein regulators. In searching for GDIs for CDC42, Leonard et al (1992) J. Biol Chem., have recently isolated and purified a protein which acts as a strong GDI for CDC42. Based on cyanogen-bromide-generated sequence, the CDC42-GDI appears to be very similar, if not identical to, the rhoGDI. In addition, a common GDI appears to couple to the rac 1 protein (as well as to rho and CDC42Hs). Given the sequence similarity between D4 and the rho-subtype GDI, the iD4 protein was tested for GDI activity.

Figure 10A is the principle of the GDI assay. Figures 10B, 10C and 10D show that D4 exerts a low level of GDI activity against CDC42 and rac 1. Compared to an equivalent quantity of rhoGDI, it was estimated that D4 has only about 5-10 % of the GDI activity against the target G-proteins. The dose response profiles indicate that D4 binds significantly more weakly but has activity has a GDI.

GDIs, by virtue of their function, are rate limiting factors in the generation of activated G-proteins and are therefore potential tumor

suppressors. In this context, the mapping of D4 to chromosome 12p 12-13 is relevant. A significant percentage of acute leukemias contain either deletions or translocations involving this region. Deletions of 12p 12-13 in acute leukemias have been described by Carroll, et al., (1987) Blood 70:1962-1965. A non-random t(4;12p12) translocation has also been described by Raimondi, et al., (1991) Blood 77:2016-2022). Since D4 has GDI activity, it is possible that D4 is involved in leukemogenesis. Further understanding of the significance of D4 in hematopoietic cells can be gained by examining the affect of D4 mutations and deletions in cell lines and animals.

A comparison of the sequence of murine and human D4 shows that the similarity with rhoGDI is distributed across the molecule. The greatest divergence is in the first 20 or so amino acids. Despite this similarity, the functional studies of D4 show that it is capable of exerting GDI activity at a level many times lower than rhoGDI against the same target GTP binding protein, CDC42Hs and rac 1. There are two possible explanations. It is possible that D4 is a GDI for one of these known molecules. For example, rac 2 is specifically expressed in myeloid cells and lymphoid cells. However, rac 2 is almost identical to rac 1, differing only by a few amino acids. It is very doubtful that D4 will exhibit a more significant GDI activity against rac 2. Rho and rhoGDI are expressed widely, and in more or less the same levels, in most hematopoietic and non-hematopoietic cells. In contrast, D4 is clearly preferentially expressed at very high levels in hematopoietic cells. Thus, it seems unlikely that D4 acts as a redundant GDI for rho. Finally, there is no similarity at all between D4 and the smg25(rab) GDI. As a result, it is believed that D4 is either

a GDI for an as yet unknown GTP-binding protein or that the real *in vivo* function of D4 is not the inhibition of GDP dissociation.

5 **Comparison and substitution of GDI activity of rhoGDI and D4.**

Assays with both murine and human D4 against CDC42, rac I and ras revealed that D4 has detectable GDI activity against CDC42 and rac 1 and not ras. However, to obtain the same amount of inhibition as rhoGDI, at least ten times more concentration of D4 protein is required, as shown by Figure 11 (compare activity curve of GST-mD4 with GST-GDI). The increasing GDI activity of D4 with the increasing amount of the fusion protein demonstrates that D4 can function as a GDI, and that the binding of D4 to CDC42 and rac I is much lower, by about 30-40 times, than rhoGDI. The phenomenon of a partial GDI activity is very unusual and has not been seen before. This suggests that the natural substrate of D4 is not GDI or that the assay is not performed with the right GTP-binding protein. It is likely that D4 regulates a completely novel GTP-binding protein.

As described below, experiments and observations using chimeric proteins of D4 and rhoGDI demonstrated that the C-terminus of rhoGDI determines specificity for GDI binding to rho protein, and that this specificity can be conferred on D4 by substitution of the C terminal region of rhoGDI for D4 and vice versa.

Construction of the rho GDI deletion mutants. The GST-GDI plasmids encoding carboxyl terminal truncations of 4 and 8 amino acids, and an amino terminal truncation of 25 amino acids were all constructed by the polymerase chain reaction (PCR). The template used was pGST-GDI, which expresses rho GDI as a fusion protein with glutathione-S-transferase (GST), as described by Leonard, et al., (1992) J. Biol. Chem. 267, 22860-22868. The

oligonucleotides CGAAGCTTTCAGAGATTCCACTCCCA and CGAAGCTTTCAGAGATTCCACTCCCA were designed to introduce a stop codon after amino acid residues 196 and 200, respectively, in the rho GDI coding sequence. This primer also contains a *HindIII* restriction enzyme site directly following the stop codon. An oligonucleotide (CTGGTTCGCGTGGATC) was also generated to anneal to the *BamHI* restriction site just upstream (5') from the start of the GDI coding sequence in the pGST-GDI plasmid. The 5' and 3' primers for the 25 residue amino terminal truncation were GCGAATTCATTATAAGCCGCCGCCAGAA and TGAATTAAGCTTGAGCT, respectively. PCR was performed using these primers and the pGST-GDI plasmid according to the conditions recommended by Perkin-Elmer/Cetus except for the addition of 20% glycerol into the reaction mixture. The PCR products and pGEX-KG plasmid (Guan and Dixon, (1991) Anal. Biochem. 192, 262-267.) were digested with *EcoRI* and *HindIII*, ligated together, and then electroporated (200 ohms, 2.5 μ FD, 2.5 V) into JM101 *E. coli*. Plasmid minipreps of single colonies of all constructs were sequenced and found to contain the proper truncations.

Construction of the pGST-LD4, pGST-mD4 and the chimeric plasmids. The constructs encoding GST fusion proteins of LD4 and its murine homolog, mD4 (pGST-LD4 and pGST-mD4, respectively) were prepared in the same way, except for the specific template and primers used for PCR. For LD4, the 5' primer was GCTCTAGAGCTTATGACTGAA, the 3' primer was GCGTCGACTCATTCTGTCCACTCCT, and the template was the pCA1040 plasmid containing the cDNA for LD4 (Lelias, et al., (1993) Proc. Natl. Acad. Sci. USA 90, 1479-1483). For mD4, the same 3' primer was used, while the 5' primer was GCTCTAGAGCTTATGACGGAGAAGGAT, and the template was the murine D4 cDNA described above.

PCR was performed and the products were digested with *Xba*I and *Hind*III and ligated into the pGEX-KG vector which had been digested with the same enzymes.

5 The pGST-GDI and pGST-LD4 plasmids described
above were then used as templates to amplify DNA
encoding 84% of the full length GDI and LD4 sequences
(starting from the amino terminus). In each case,
the 5' primer was CTGGTTCGCGTGGATC, and the 3'
10 primer was CGAAGCTTTCAAGCTAGCATGCCCTT. The DNA
encoding the carboxyl terminal portions of the rho
GDI and LD4 proteins (which comprised 16% of their
full length sequences) was amplified using the same
t e m p l a t e s , a n d t h e p r i m e r s
GCGAATTCTGCCCAAGGGCATGCTAGC (5') and
15 TGAATTAAGCTTGAGCT (3'). The resulting PCR products
were digested with *Eco*RI and *Hind*III and ligated into
the pGEX-KG vector digested with the same enzymes.
All partial chimeric plasmids (ie. those that
contained only the carboxyl terminal or amino
20 terminal portion of one of the genes) were digested
with *Eco*RI and *Nhe*I. The products containing the
amino terminal portion of the cDNA were purified and
then ligated into the plasmid containing the carboxyl
terminal regions to generate the hybrid genes GST-G_NL_C
and GST-L_NG_C. All constructs were sequenced and found
25 to contain the proper insertions.

Expression and purification of wildtype and
mutant fusion proteins. The GST-GDI, GST-LD4, GST-
mD4, and all chimeric and truncated proteins were
30 expressed and purified as described by Leonard, et
al. Fractions of pure fusion protein were either
used immediately (in the case of the deletion
analysis) or after storing at -20°C (in the case of
the analysis of the chimeric proteins and GST-mD4).
35 Proteins that contained the amino terminus of LD4
(i.e. GST-LD4 and the chimera, GST-L_NG_C were sensitive
to *E. coli* proteolytic enzymes so that as much as 50%

5 of the total protein was present as proteolyzed fragments (with the remaining 50% representing the parent molecule). All other fusion proteins showed very little proteolysis such that greater 95% of the total protein existed as the parent molecule as judged by SDS-PAGE. Protein concentrations were determined by Bradford assay using rabbit IgG (Sigma) as the standard.

10 *GDP dissociation inhibition assays and membrane dissociation assays.* The effects of the fusion proteins on the dissociation of radiolabeled GDP from Cdc42Hs were assessed by filter binding assay as described by Leonard, et al., except that the amount of platelet-purified used in each assay was about 7
15 ng. The fusion proteins were tested for their ability to release membrane-bound Cdc42Hs into the soluble phase, except that membranes were prepared from *S. frugiperda* insect cells infected with a recombinant baculovirus encoding the Cdc42Hs protein.
20 After incubation of the membranes (containing about 9 ng of Cdc42Hs) with the indicated amount of the fusion protein for 25-30 minutes (this time was constant for any set of comparison assays), the membranes were centrifuged for 10 min in an Eppendorf
25 microcentrifuge tube at 16,000 x g. The Cdc42Hs released from the membranes was determined by quantitative Western blotting using ¹²⁵I-protein A.

Results

30 Deletion of the last four amino acids in rhoGDI does not affect rhoGDI activity. However, deletion of the last eight amino acids abolishes completely the GDI activity of rhoGDI. To probe further the importance of the C terminal domain of rhoGDI and D4,
35 chimeric proteins between D4 and rhoGDI were constructed and expressed. Figure 11 shows that by replacing the C terminal 33 amino acids of D4 with that of rhoGDI, the chimera functions as effectively

as rhoGDI. Likewise, when the C terminal 33 amino acids of rhoGDI is replaced with that of D4, the chimeric rhoGDI functions like D4. To further test the importance of the carboxyl terminal domain of the GDI molecule, chimeras were constructed between rho GDI and the related LD4 protein which is 10 to 20-fold less potent than rho GDI in functional assays with the Cdc42Hs protein. A chimera that contained the amino terminal 168 amino acids of LD4 and only 33 carboxyl terminal amino acids of rho GDI fully mimicked the wild type rho GDI in its ability to inhibit GDP dissociation and release Cdc42Hs from membranes. The corresponding rho GDI/LD4 chimera that contained the entire rho GDI sequence except that the carboxyl terminal 33 amino acids were from LD4 behaved more like the wild type LD4 and was at least 20 fold less effective than rho GDI. Overall, these results highlight a domain that is responsible for imparting functional specificity in a key regulator of rho-type GTP-binding proteins. This leads to the conclusion that the carboxyl domain of the rho GDI is responsible for functional specificity.

The final 33 residues of rho GDI and LD4 are very well conserved between these two proteins. Residues 196-200 in rho GDI, which when deleted result in a complete loss of activity, are almost identical to the corresponding residues in LD4, with the only difference in these four amino acids being Thr¹⁹⁷ (rho GDI) and Ser¹⁹⁴ (LD4). It seems unlikely that this conservative change would be responsible for the large effects on the dose responses. Rather, it seems more likely that this region may be necessary to stabilize the structure of another interaction domain, possibly located upstream from these residues within the carboxyl terminal 16% of the protein, as suggested by the chimera studies.

One of the most apparent differences between rho GDI and LD4 within this region occurs at positions 169-170; in LD4 the two residues at these positions are QD whereas in all other family members including the murine homolog of LD4, mD4, the residues are RG. To determine if these residues were responsible for the weaker activity of LD4, GST-mD4 was examined in the GDP dissociation inhibition assay. The dose response profile obtained for GST-mD4 was very similar to that obtained for GST-LD4, with the apparent EC_{50} values being 7-8 μ M. Thus, it is concluded that the QD residues are most likely not responsible for the lower activity of LD4. These results also suggest that D4 might have a specific substrate which has not yet been identified.

Intracellular localization of D4 with epitope tagging.

The accumulating evidence is that D4 might have properties quite different from rhoGDI. To more clearly ascertain whether or not D4 may be involved in the cell cycle, the intracellular localization of D4 was determined. The epitope-tagging method was used, which makes use of tagging specific epitopes to either end of the protein of interest (D4), which is then expressed in cells and localized by monoclonal antibodies against the epitopes by an *in situ* immunofluorescent technique.

The D4 protein was tagged with the c-myc epitope (EQKLISEEDL). CMV-promoter driven mammalian expression vectors containing the myc epitopes were obtained from Dr. Frank McKeon (Harvard Medical School). PCR amplified human D4 cDNA was cloned in-frame 3' to the epitope to give the ^{myc}D4 vector. The construct was sequenced to ensure no mutation was introduced.

To check whether the ^{myc}D4 vector will express the expected protein, vector DNA was transfected into

the baby hamster kidney (BHK) cell line described by Heald and Mckeen using a calcium precipitation technique. Previous studies with murine normal tissues showed that D4 mRNA is detectable in kidney total RNA. A myc-tagged cyclophilin B construct (mycCypB) and the myc-epitope vector DNAs were transfected as positive and negative control respectively. After an overnight incubation to recover, transfected cells were metabolically labelled with ^{35}S -methionine for 5 hours. The cells were lysed with 50 mM Tris, 100 mM NaCl, 0.4% SDS, the supernatant immunoprecipitated overnight with anti-myc epitope monoclonal antibody (9E10), immunocomplexes isolated by protein G-agarose (Sigma) and fractionated on SDS-10% PAGE and analyzed by Phosphorimager (Molecular Dynamics).

Specific proteins of about 23 kd for mycCypB and 28.5 kd for mycD4 were isolated for each myc-tagged protein; no protein was seen when the myc-epitope vector alone was transfected.

BHK cells grown on 18 mm round coverslips were transfected with 2 μg of mycD4 plasmid DNA for 4 hours and allowed to recover for 12 hr. Cells were fixed with 3% formaldehyde in PBS, washed with 0.1% NP-40 in PBS to permeabilized the cells. 9E10 monoclonal antibody (mAb) was incubated on the coverslips for 30 min., washed and followed by rhodamine-conjugated goat anti-mouse secondary antibody. DNA was labelled with Hoechst dye 33258 (Sigma) at 1 $\mu\text{g}/\text{ml}$ in PBS. Coverslips were mounted on glass slides in 90% glycerol and 10% 0.2 M Tris (pH 8). Immunofluorescent detection of myc-D4 protein with 9E10 anti-myc-epitope antibody showed that some cells (44%) manifest diffuse punctate distribution of protein only in the cytoplasm with total exclusion of protein from the nuclei. Some cells (25%) show simultaneous presence of protein in cytoplasm and

nucleus. In a certain population (31%), D4 protein was localized strictly to the nuclei. The specificity of the antibody is demonstrated by the presence of negative cells in the same field.

5 It was determined whether or not over-expression of protein could lead to artefactual localization. For example, some proteins might migrate to the nucleus because of small size (less than 20 Kd) and overflowing from the cytoplasm. Since the D4 protein is between 27 and 28 Kd Mw, this would argue against spontaneous overflowing into nuclei. Furthermore, in cells where there is clearly a very high level of protein in the cytoplasm, nuclei staining were negative. In contrast, in cells where staining was seen only in nuclei, the amount of total protein was clearly less than those in cells with pure cytoplasmic staining. Therefore, the strongest argument that this is not an artefact is because a distinctly different distribution of D4 protein is observed, leading to the conclusion that the localization of D4 to the nucleus in some cells involves nuclear events.

D4 can act as a suppressor of transformation.

25 D4, by functioning as a negative regulator of activation of ras related GTP-binding proteins through its GDI activity, might prevent the process of transformation. This potential tumor suppression capacity of D4 was determined by simultaneous comparison with a conventionally accepted anti-oncogene, in an assay described by Finlay et al. in their demonstration of p53 as a tumor suppressor. This involves co-transfection of two oncogenes with the p53 wild-type (wt) gene into primary rat embryo fibroblasts (REF).

35 REF were prepared from 14-15 day old Fisher rat embryos. Only early-passage (P4 or less) were used. A transfection mixture contained 3 μ g each of two

transforming DNA (ras, E1A or p53 mutant, kindly provided by Dr. Peacocke, Tufts Medical School) with or without 9 μ g of p53 wild-type (wt), D4 cDNA in an expression vector and salmon sperm as carrier DNA to bring total DNA in mixture to 15 μ g. ras is the MoLTR-ras plasmid containing the transforming murine Harvey ras sarcoma virus, mutant codon 12, driven by the Moloney sarcoma virus LTR. E1A contains the Adenovirus type 5 E1A EcoR1-SacI fragment with the promoter replaced by SV40 enhancer-promoter. p53 mutant (m) plasmid is a cDNA/genomic hybrid for murine p53, with the val₁₃₅-mutation, driven by the Harvey murine sarcoma virus LTR. p53 wild type (wt) plasmid is similar to p53m except it contains the wild type alanine₁₃₅. Transformed foci were scored 14-18 days later. The number of foci obtained by transfection of two oncogenes is compared with the foci obtained when the gene to be tested is included as the third DNA.

Figures 12A, 12B, 12C and 12D show the data from several experiments testing the ability of D4 as a tumor suppressor, as compared with the known tumor suppressor p53wt. Figure 12A shows the reduction of foci formed by ras plus E1A when p53wt is cotransfected (1:1:3 DNA). When human D4, driven by pgk or CMV promoter, is used, the suppression of foci was even more. Figure 12B shows that CMV-D4 suppressed foci formation by E1A plus p53m as well as p53wt. Figure 12C shows that when ras plus p53m were used, D4 appeared not to be as effective as p53wt. However, transformation was still suppressed by more than 60%. Figure 12D shows an experiment using different quantities (3, 9 or 12 μ g) of CMV vector (CMV_v), p53wt or CMV-D4 against ras plus E1A (3 μ g each). These results show that (i) excess CMV_v DNA might slightly reduce transformation efficiency; (ii) within every dose group, the same degree of foci

suppression by CMV-D4 is seen.

These results together argue very strongly that D4 might have properties of a tumor suppressor. They also indicate that D4 has functions related to cell cycling.

Clinical, Diagnostic and Research application.

D4 has properties of a GDI and is preferentially expressed in hematopoietic cells. As a GDI, D4 should play a key role in regulating the activity of these GTP-binding proteins. The current view is that GDIs, among other cellular functions, are potential tumor suppressors. Physiologically, GDIs, such as D4, are crucial in the proper control of cell division and differentiation. The identification of these molecules opens up an area accessible to therapeutic intervention (Marx J. (1992) Science 257:484-485). Available information indicates that D4 very likely plays an important role in the differentiation and proliferation of hematopoietic cells and perhaps in a few other tissues as well, such as brain, pre-adipocytes, muscle cells, melanocytes, lung and prostate.

The super family of ras related small GTP-binding proteins, as described in more detail below, are involved in a wide range of important cellular functions. D4 could also turn out to be an important regulator of different normal physiological functions of a specific lineages.

The super family of ras/ras-like genes

ras Gene: Genes of the ras family (H, K and N-ras) were first identified as transforming genes in human tumors by Barbacid M. (1987) Ann. Rev. Biochem. 56:779-827. The high degree of conservation of ras genes throughout eukaryotic evolution indicates that they very likely have a fundamental role in basic cellular functions. The ras proteins are similar to adenylate cyclase G-proteins in binding GTP, having

GTPase activity and localizing to the inner leaflet of the plasma membrane, Kaziro Y, et al. (1991) Annual Review of Biochem 60:349-400. Much evidence indicates that the ras gene products participate in transducing across cellular membranes signals that regulate cell division, Hall A. (1992) Cell 69:389-391; McCormick F. (1989) Cell 56:5. Its implication in leukemogenesis is substantiated by the discovery of ras mutations in MDS, Hirai H, et al., (1987) Nature 327:430-432, and acute leukemias, Bos, et al., (1985) Nature 315(27):726-730.

rho, ras-related gene family: Recently, another class of ras-related genes has been identified, adding further complexity to the research on ras. This new gene family, denoted rho for ras homology, encodes proteins that share 35% amino acid homology with ras, Madaule P, Axel R. (1985) Cell 41:31-40. Three highly homologous members of the rho family, A, B and C, have been identified. The rho proteins exist widely in various species, from yeast to man, as described for ras p21s, Madaule P, Axel R, Myers AM. (1987) Proc. Natl. Acad. Sci. USA 84:779-783. As in ras, rho is highly conserved evolutionarily, with the aplysia (snail) rho and human rhoA sharing 85% protein identities, Madaule P, Axel R. (1985) Cell 41:31-40. Since the discovery of the rho family, several other ras-related G-proteins have been identified, all with the Ras canonical boxes. They can be divided into four major groups based on protein sequences: (i) the ras group that includes the proto-oncogenes H-, K- and N-ras (Ellis, et al. (1981) Nature (London) 292:506-511; Lowe, et al. (1987) Cell 48:137-146), ral (Chardin P, Tavitian. (1986) EMBO J. 5:2203-2208), and rap (Pizon, et al., (1988) Oncogene 3:201-204) genes, (ii) rab or YPT group (T uchot N, Chardin P, Tavitian A. (1987) Proc. Natl. Acad. Sci. USA 84:8210-8214), (iii) the rho

group which includes CDC42, rac I and II, and TC10 (Vincent S, Jeanteur P, Fort P. (1992) Molecular and Cellular Biology 12(7):3138-3148), and (iv) TC4 (Drivas, et al., (1990) Mol. Cell. Biol. 10:1793-1798).

The functions of these ras-related proteins remain unclear. Several experiments have illustrated the striking effect rho proteins have on cellular functions. Disruption of yeast rho 1 gene is lethal for the microorganism (Madaule and Axel (1985) Cell 41:41-40). Overexpression of the rho A gene in fibroblasts reduces serum dependence for cell growth and is tumorigenic in nude mice (Avraham H, Weinberg RA. (1989) Mol. Cell Biol. 9:2058-2066). Ribosylation of rho proteins by ADP-ribosyltransferase C3 induces morphological changes of several types of cells through microfilament disassembly, as reported by Chardin, et al. (1989) EMBO 8:1087-1092; Ridley AJ, Hall A. (1992) Cell 70:389-399. More recently, Ridley et al have shown, by injection of rho and rac protein into fibroblasts, that rho controls the formation of stress fibers (Ridley, et al. (1992) Cell 70:401-410), while rac causes the ruffling of cells (Trahey, et al. (1988) Science 242:1697-1700). Thus, it is apparent that rho proteins are involved in the regulation of cytoskeletal organization and cell morphology. The whole family of rho related proteins contain several domains with stretches of identical residues, as reviewed by Vincent S, Jeanteur P, Fort P. (1992) Mol. Cell. Biol. 12(7):3138-3148.

Regulators of GTP-binding/GTPase cycles of ras-related G-proteins. The small G-proteins bind to guanine nucleotides (GTP and GDP) and possess low intrinsic GTPase activity. Three classes of regulators are recognized so far, as depicted in Figure 13: the GTPase-activating proteins (GAPs),

stimulators of GDP dissociation (GDS) and the inhibitors of GDP-dissociation (GDI). Several such regulators have been isolated for different members of the ras-super family. It turns out that ras-GAP (Trahey, et al. (1988) Science 242:1697-1700; Vogel, et al., (1988) Nature 335:90-93), is a member of a family of GAPs with a highly conserved domain and include the neurofibromatosis(NF1) gene product (McCormick F. (1989) Cell 56:5), the yeast IRA proteins (Tanaka, et al., (1990) Cell 60:803-807), and GAP 1 (Imai, et al., (1991) Mol. Cell. Biol. 11, 3088-3094). Another group of GAPs is linked by a different homologous domain, including rhoGAP, chimerin, PI-3 kinase subunit p85a,b (Garrett, et al., (1989) J. Biol Chem 264:10-13). Similarly, a family of GDS exists: DBL (Borzillo, et al., (1990) Mol. Cell Biol. 10:2704-2174), VAV (Katzav, et al. (1989) EMBO Journal 8:2283-2290), and LBC, recognized by a homologous domain required for transformation in hematopoietic cells. Two GDI have been described so far, rab3/smgp25a-GDI (Matusui, et al., (1990) Mol. Cell. Biol. 10:4116-4122), and rho-GDI (Ueda, et al., (1990) J. Biol. Chem. 265:9373-9380; Fukumoto, et al., (1990) Oncogene 5:1321-1328), and there are no similarities between them.

Biochemical connection between ras and other ras-related proteins. Ras and ras-like proteins interact through complex molecules with multiple functional G-protein-interacting domains. The cDNA for the rasGAP-binding protein pl 90 (Ellis, et al., (1992) Nature 343:377-381), was cloned recently and found to have a GTP-binding domain, a transcription repressor domain and the rhoGAP homologous domain (Settleman, et al., (1992) Cell 69:539-549). BCR is a large pl 60 protein with a novel serine-kinase domain (Maru Y, Witte ON. (1991) Cell 67:459-468), a dbl-homologous domain(GDS) (Ron, et al., (1991) New

Biol. 3:372), and a rho-homologous GAP for p21rac
 (Diekmann, et al., (1991) Nature 351:400-402). The
 recently cloned rasGDS has the CDC25 homologous GDS
 domain in the C-terminus while the amino end contains
 5 the dbl-homologous domain (Shou (1992) Nature
 358:351-354). Common to many of these are the
 SH2/SH3 motifs (e.g. in vav, rasGAP, Pl-3 kinase
 subunit) believed to be important for the linking of
 10 activated receptors to downstream signals (Koch, et
 al., (1991) Science 252:668-674; Hall A. (1990)
 Science 249:635-640; Puil L, Pawson T. (1992) Cell
 Regulation 2:275-277). At the moment, however, one
 can only speculate that an intimate biochemical
 15 relation exists between the ras superfamilies, with
 ras predominantly controlling the cell cycle and the
 rho-family of protein regulating cell morphology and
 other physiological functions. However, there is
 increasing evidence pointing toward many critical
 20 roles played by the super-family of ras-related small
 GTP-binding proteins in the cellular differentiation,
 proliferation and functions of a wide range of
 tissues. Regulators of the active state of G--
 proteins are therefore critical molecules. This
 25 importance is underscored by increasing evidence
 implicating them in clinically important diseases.
 The dbl proto-oncogene, identified initially as an
 oncogene in a human B-cell lymphoma, is a guanine
 nucleotide exchange factor for the ras-related
 30 protein, CDC42Hs (Hart, et al., (1991) Nature
 354:311-314). Bcr, the breakpoint cluster region
 gene involved in chronic myeloid leukemia, encodes a
 multi-functional protein that include a
 dbl-homologous domain (Ron, et al., (1991) New Biol.
 3:372), and a domain for activating GTPase activity
 35 (GAP) of p21rac (Diekmann, et al., (1991) Nature
 351:400-402). Neurofibrin (NF1), the
 neur fibromatosis type 1 susceptibility gene, is a

GAP protein for p21ras (Bollag G, McCormick F. (1992) Nature 356:663-664).

5 The preferential expression of D4 in blood cells is indicative of a gene with unique and functional significance in hematopoietic cells. It is interesting to note that vav (Katzav, et al., (1989) EMBO Journal 8:2283-2290), a proto-oncogene expressed specifically in all hematopoietic lineages, contains a region sharing homologies with the GDP/GTP exchange domain of db1, CDC24 and bcr (Adams, et al., (1992) Oncogene 7:611-618). These proteins may represent a related family of positive regulators acting as catalysts for the dissociation of GDP from G-proteins in different tissues. It is very likely that a group of GDP-dissociation inhibitors, such as rhoGDI and possibly D4, function as negative regulators of G-protein activation. The determination of the biochemical action of D4 is therefore of great interest and expected to be very important in the role cancer and cell differentiation, especially of hematopoietic cells.

Diagnostic Application:

25 i) Leukemia. D4 cDNA can be used to analyze RNA and DNA from leukemic patients, particular those with chromosomal abnormalities involving chromosome 12p 12-13 region, and other diseases suspected to involve D4. RNAs will be examined for missing or aberrant transcripts of D4 and DNAs will be examined for rearrangements of the D4 gene. This will help scientists and clinicians determine if there is a sub-type of leukemia due to abnormalities of the D4 gene or its expression.

35 Other Diseases. Other useful areas of diagnostic application include studies of diseases of tissues where D4 is expressed but at a low level, including lung, skin (melanocytes), prostate, muscle, kidney, ovaries, bladder, fatty and neuronal tissue.

The D4 cDNA can be used in diagnosis using standard techniques. In most cases, it is necessary to use only short sequential sequences of the cDNA or gene, ranging from seventeen to thirty oligonucleotides in length. The RNA can also be used. The nucleic acid can be screened for hybridization as described above or labelled with a dye, fluorescent molecule, radiolabel or enzyme for detection, or immobilized and used in that manner for screening.

Clinical Application:

Use of the Protein:

i) Treatment of leukemia and Other Diseases.

In those diseases where the D4 gene is defective or the protein missing, D4 will be administered into, or the D4 gene introduced into, the cells where the protein is missing or defective. Although the delivery of specific proteins into cells is still a major obstacle in the use and application of many proteins which could now be produced in industrial scale if necessary, several methodologies are being explored to achieve intra-cellular delivery of intact proteins. These methodologies include:

a) Polyethylene Glycol (PEG)-modified protein.

The technique is based on the principle of increasing the plasma half-life and reducing the immunogenicity of circulating protein by a covalent conjugation of the protein to PEG. The best example is the successful treatment of patients with the Adenosine Deaminase (ADA) enzyme deficiency disease, a Sub-acute Combined Immunodeficiency (SCID), with the PEG-ADA, as reported by Hershtfield, et al., (1987) New Eng. J. Med. 316:589-596. This has encouraged exploration of more versatile uses of PEG such as conjugation to growth factors (Tanaka, et al., (1991) Cancer Research 51:3710-3714), antibodies (Kitamura, et al., (1991) Cancer Research 51:4310-4315),

chemotherapeutic agents and modifying proteins to increase additional sites for PEG attachment (Hershfield, et al., (1991) Proc. Natl. Acad. Sci. USA 88:7185-7189).

5 b) Liposomes. The use of liposomes to deliver drugs and macromolecules to tissues *in vivo* is being intensely pursued, as reported by Ostro, MJ, ed. (1989) Liposomes from Biophysics to Therapeutics (Dekker, New York) pp. 1-369; Gregoriadis, G. ed. 10 (1988) Liposomes as Drug Carriers (Wiley, New York), pp. 1-863. The method has the potential of delivering a wide range of drugs and macromolecules, including recombinant proteins. Modifications of the lipid composition have generated carriers that not 15 only avoid rapid uptake by the reticulo-endothelial system but can be targeted to certain tissues (Papahadjopoulos, et al., (1991) Proc. Natl. Acad. Sci. USA 88:11460-11464; Maruyama K, Kennel S, Huang L. (1990) Proc. Natl. Acad. Sci. USA 87:5744-5748; 20 Hughes BJ, Kennel, Lee R, Huang L. (1989) Cancer Research 49:6214-6220). *In vivo* trials have also demonstrated the feasibility of clinical applications, as reported by Lopez-Berestein, et al., (1985) J. Inf. Dis. 151:704-710.

25 c) Receptor-mediated endocytosis of toxins, such as diphtheria and pseudomonas toxin conjugated to natural ligands such as polypeptide hormones, has been exploited successfully to deliver macromolecules to certain cells, as reported by Vitetta, et al., 30 (1987) Science 238:1098-1104; Pastan, et al., (1986) Cell 47:641-648. This methodology is being explored further to expand the repertoire of potential target tissues. An example is the recently described use of the endocytosis of folate-conjugated proteins, 35 through the folate receptor present and active in all dividing cells, to deliver large number of intact macromolecules (greater than 10^8 copies) into cells,

as reported by Leamon CP, Low PS. (1991) Proc. Natl. Acad. Sci. USA 88:5572-5576.

If a sub-type of leukemia with an abnormality of the D4 gene is identified, the patient can be treated by providing with or administering to the malignant cells the functional protein. The same methodology can be applied to defects or diseases of other tissues which involves defects in or absent D4.

Use of the cDNA/Gene:

i) In vitro Amplification of Hematopoietic Stem Cells. There is substantial preliminary evidence indicating that the need for D4 is greatly increased during the growth and differentiation of very primitive hematopoietic progenitor cells. It is therefore believed that D4 is necessary for the progression of differentiation and that blocking the activity of D4 might stop differentiation but not proliferation.

Currently there is considerable effort being invested to devise a means of harvesting bone-marrow from patients, amplify the number of stem cells (before transplantation) by in vitro culture and stimulation with various combinations of the presently known plethora of hematopoietic cytokines. One of the major problems encountered is that many of the cytokines stimulate division and differentiation at the same time. By blocking the activity of D4 it may be possible to temporarily inhibit the differentiation but not the amplification. One way of achieving this is to block the translation of the protein with antisense oligo nucleotides (Wagner, et al., (1992) Proc. Natl. Acad. Sci. 89:6785-6789; Skorski, et al., (1992) J. Exp. Med. 175:743-750), based on the sequence information of D4. This technique is already moving rapidly into in vivo animal experiments with practical clinical application in the not too distant future, as

reported by Bayever, et al., (1992) Blood 80, Suppl 1, Abstract 833.

5 ii) Gene Therapy. The replacement of a defective gene in a cell with a normal gene is already being tested in clinical trials, as reported by Miller AD. (1992) Nature 357:455-460. 'Gene therapy' has been successfully achieved for a few cases of SCID patients who are being closely followed. It is expected that this means of therapy will be applicable for a wide range of genetic diseases involving different tissues. A dramatic example is the *in vivo* transfer of the human Cystic Fibrosis transmembrane conductance regulator gene to the airway epithelium of rats, as reported by Rosenfeld, et al., (1992) Cell 68:143-155.

10 The D4 cDNA can also be used for gene therapy of disorders involving absent or defective D4 using retroviral vectors (Miller AD. (1992) Nature 357:455-460; van Beusechem, et al., (1992) Proc. Natl. Acad. Sci. 89:7640-7644), adenoviral vectors (Rosenfeld, et al., (1992) Cell 68:143-155; Lemarchand, et al., (1992) Proc. Natl. Acad. Sci. 89:7640-7644), and physical methods of DNA and DNA-complexes transfer via liposomes (Miller AD. (1992) Nature 357:455-460), and transferrin and other cell specific receptors (Zenke, et al., (1990) Proc. Natl. Acad. Sci. USA, 87:3655-3659; Curiel, et al., (1991) Proc. Natl. Acad. Sci. 88:8850-8854; Wu, et al., (1991) J. Biol. Chem. 266:14338-14342).

15 **Research Application:**

20 i) Use of Specific Antibody. Rabbits were immunized against the D4 protein. The antibody isolated from the serum is capable of identifying a specific protein band, corresponding to the D4 protein, in hematopoietic cells. The antibody can be used to look for interacting protein(s) that can be identified by the co-immuno-precipitating technique

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of Settleman, et al., (1992) Cell 69:539-549; and Wong, et al., (1992) Cell 69:551-558. The antibody can also be used for diagnostic purposes, e.g., examination of tissues. A number of screening techniques are well known for the use of antibodies alone, or labelled with a dye, fluorescent label, radiolabel, or enzyme.

ii) Use of the Protein. The D1 protein can also be used to look for interacting proteins using in situ hybridization techniques which have been successfully used in identifying several important biological molecules, as reported by Blonar MA, Rutter WJ. (1991) Science 256:1014-1018; LeClair KP, Blonar MA, Sharp PA. (1992) Proc. Natl. Acad. Sci. USA, 89:8145-8149; Cicchetti P, Mayer BJ, Thiel G, Baltimore D. (1992) Science 257:803-806; Kaelin, et al., (1992) Cell 70:351-364.

iii) Animal Models with D4 Defect. The genomic clone of D4 has been isolated. This can be used to create a gene-targeting vector to disrupt the D4 gene in murine embryonal stem (ES) cells by homologous recombination, using the method of Capecchi MR. (1989) Science 244:1288-1292; Koller BH, Smithies O. (1992) Ann. Rev. Immunol. 10:705-730. Such cells can then be used to generate animals that are either heterozygous or homozygous for D4 gene deletion, using the method of Travis J. (1992) Science 256:1392-1394. These animals will serve as excellent models for biological and pharmacological investigations in the field of GTP-binding proteins.

The teachings of the foregoing publications are illustrative of the skill and techniques known to those skill in the art and are not believed to constitute essential information. The teachings of the publications are specifically incorporated by reference.

Modifications and variations of the D4 protein

and DNA encoding the protein will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Cytomed
(B) STREET: 840 Memorial Drive
(C) CITY: Cambridge
(D) STATE: Massachusetts
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 02139
(G) TELEPHONE: (617)-661-3400
(H) TELEFAX: (617)-661-7364

(A) NAME: Beth Israel Hospital Association
(B) STREET: 330 Brookline Avenue
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 02215
(G) TELEPHONE: (617)-735-4585
(H) TELEFAX: (617)-735-4774

(A) NAME: Bing Lim
(B) STREET: 4 Hooper Street
(C) CITY: Dorchester
(D) STATE: Massachusetts
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 02124

(A) NAME: Jean-Michel Lelias
(B) STREET: 41 Rue Louise de Karoual
(C) CITY: Evry
(E) COUNTRY: France
(F) POSTAL CODE (ZIP): 91000

(A) NAME: Chaker Adra
 (B) STREET: 65 East India Row, Apartment 37G
 (C) CITY: Boston
 (D) STATE: Massachusetts
 (E) COUNTRY: USA
 (F) POSTAL CODE (ZIP): 02110

(A) NAME: Jone Ko
 (B) STREET: 19 Willow Road
 (C) CITY: Sudbury
 (D) STATE: Massachusetts
 (E) COUNTRY: USA
 (F) POSTAL CODE (ZIP): 01776

(ii) TITLE OF INVENTION: D4 Gene and Methods of Use Thereof

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/990,337
 (B) FILING DATE: 10-DEC-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATTATGACTG AAAAAGCCCC AGAGCCACAT GTG

33

(2) INFORMATION FOR SEQ ID NO: 2:
(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GACTCTAGAT CATTCTGTCC ACTCCTTCTT

30

(2) INFORMATION FOR SEQ ID NO: 3:
(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1171 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 66..68

(D) OTHER INFORMATION: /function= "In-frame upstream stop
codon."

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 594..602

(D) OTHER INFORMATION: /function= "Consensus
N-glycosylation site."

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 648..656

(D) OTHER INFORMATION: /function= "Consensus
N-glycosylation site."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGTACTCAGA AGTCAGAGTT GAGAGACAGA GGCACCCCGG ACAGAGACGT GAAGCACTGA	60
ATAAATAGAT CAGAAATGACT GAAAAAGCCC CAGAGCCACA TGTGGAGGAG GATGACGATG	120
ATGAGCTGGA CAGCAAGCTC AATTATAAGC CTCCACCACA GAAGTCCCTG AAAGAGCTGC	180
AGGAAATGGA CAAAGATGAT GAGAGTCTAA TTAAGTACAA GAAAACGCTG CTGGGAGATG	240
GTCCTGTGGT GACAGATCCG AAAGCCCCCA ATGTCGTTGT CACCCGGGCTC ACCCTGGTTT	300
GTGAGAGTGC CCGGGGACCA ATCACCATGG ACCTTACTGG AGATCTGGAA GCCCTCAAAA	360
AGGAAACCAT TGTGTTAAAG GAAGGTTCTG AATATAGAGT CAAAATTCAC TTCAAAGTGA	420
ACAGGGATAT TGTGTCAGGC CTGAAATACG TTCAGCACAC CTACAGGACT GGGGTGAAAG	480
TGGATAAAGC AACATTATG GTTGGCAGCT ATGGACCTCG GCCTGAGGAG TATGAGTTCC	540
TCACTCCAGT TGAGGAGGCT CCCAAGGGCA TGCTGGCCCA AGACACGTAC CACAACAAGT	600
CCTTCTTCAC CGACGATGAC AAGCAAGACC ACCTCAGCTG GGAGTGGAAC CTGTGCGATTA	660

AGAAGGAGTG GACAGAAATGA ATGCATCCAC CCCTTTCCCC ACCCTTGCCA CCTGGAAGAA 720
 TTCTCTCAGG CGTGTTCAGC ACCCTGTCCC TCCTCCCTGT CCACAGCTGG GTCCCTCTTC 780
 AACACTGCCA CATTTCCCTTA TTGATGCATC TTTTCCCACC CTGTCACTCA ACGTGGTCCC 840
 TAGAACAGA GGCTTAAAC CGGGCTTTCA CCCAACCTGC TCCCTCTGAT CCTCCATCAG 900
 GGCCAGATCT TCCACGTCTC CATCTCAGTA CACAATCATT TAATATTCC CTGTCTTACC 960
 CCTATTCAAG CAATTAGAGG CCAGAAAATG GGCAAAATTAT CACTAACAGG TCTTTGACTC 1020
 AGGTTCCAGT AGTTCATTCT AATGCCCTAGA TTCCTTTTGTG GTTGTGTGCTG GCCCAATGAG 1080
 TCCCTAGTCA CATCCCCCTGC CAGAGGGAGT TCTTCTTTTG TGAGAGACAC TGTAACGAC 1140
 ACAAGAGAAC AAGAATAAAA CAATAACTGT G 1171

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 201 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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 Lys Glu Leu Gln Glu Met Asp Lys Asp Asp Glu Ser Leu Ile Lys Tyr
 35 40 45
 Lys Lys Thr Leu Leu Gly Asp Gly Pro Val Val Thr Asp Pro Lys Ala
 50 55 60
 Pro Asn Val Val Thr Arg Leu Thr Leu Val Cys Glu Ser Ala Pro
 65 70 75 80
 Gly Pro Ile Thr Met Asp Leu Thr Gly Asp Leu Glu Ala Leu Lys Lys
 85 90 95
 Glu Thr Ile Val Leu Lys Glu Gly Ser Glu Tyr Arg Val Lys Ile His
 100 105 110
 Phe Lys Val Asn Arg Asp Ile Val Ser Gly Leu Lys Tyr Val Gln His
 115 120 125
 Thr Tyr Arg Thr Gly Val Lys Val Asp Lys Ala Thr Phe Met Val Gly
 130 135 140
 Ser Tyr Gly Pro Arg Pro Glu Glu Tyr Glu Phe Leu Thr Pro Val Glu
 145 150 155 160
 Glu Ala Pro Lys Gly Met Leu Ala Gln Asp Thr Tyr His Asn Lys Ser
 165 170 175
 Phe Phe Thr Asp Asp Lys Lys Gln Asp His Leu Ser Trp Glu Trp Asn
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 Leu Ser Ile Lys Lys Glu Trp Thr Glu
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(2) INFORMATION FOR SEQ ID NO: 5:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1107 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA
 (iii) HYPOTHETICAL: NO
 (iii) ANTI-SENSE: NO
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Murine

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CCTTGAAGGA GCTGCAGGAG ATGGACAAGG ATGACGAGAG TCTAACCAAG TACAAGAAAA	180
CACTGCTGGG AGATGTCCCT GTGGTAGCAG ACCCAACAGT TCCCAATGTG ACTGTTACCC	240
GGCTTAGCCT TGTATGTGAC AGTGCACCAG GACCCATCAC CATGGACCTT ACTGGGGATC	300
TCGAGGCCCT CAAAAGGAT ACATTGTGTC TAAAGGAAGG CATTGAATAC AGGGTGAAAA	360
TTAACTTCAA AGTGAATAAG GATATTGTGT CTGGCCTGAA GTATGTTCAA CACACATACC	420
GGACTGGCAT GAGAGTGGAT AAAGCCACAT TCATGGTTGG CAGCTATGGG CCCCAGACCAG	480
AGGAGTACGA ATTCCTCACT CCAGTAGAGG AAGCTCCCAA GGGCATGCTG GCCCGAGGCA	540
CTTACCACAA CAAGTCCTTC TTCACGGATG ACGACAAACA GGACCACCTC ACCTGGGAAT	600
GGAACCTGGC CATTAAGAAG GATTGGACAG AATGAGTGCG TCTGTCCGTC CCTCCTGTCA	660

CCTTCCTCAC CCACCAGAAG AGTTCCTCCG ACCATGTTGA TCATCACAAA CTCCTCCTCCTC 720
 CCTCCCTGTT CCTAGCTAGG CCCTTTCTCT GTCACACACA CACACACACA CATTTTCATCA 780
 CCAATATGTT TTATCTTACC CCATCTCTCA GAGTGTTCCTC TGCAAAATGAG ACTTAAAAACC 840
 CTGGCTTCTC CCACCTTGAG TCCTGAAGGT CAAGAAATGG GCAAGCTCCG GCTGACGCCCT 900
 CCTTGGCCTT CTGTTTCAGAA GTTTATTCTT GCCACTGGGT TCTTCCGGTT CGATGAACCC 960
 CGTTGTCTTC TCTGCCAAGG GGAGCTCTCC TTTTGTGGGA GACACTGTAA ACAACACCAA 1020
 AGGAAAAGAA TAAATCGTT GTGTGTGTGA CTGAGAACCT CATTTGCTTT CCTTTGCCCT 1080
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(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 201 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Murine
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Thr Glu Lys Asp Ala Gln Pro Gln Leu Glu Ala Asp Asp Asp
 1 5 10 15
 Leu Asp Ser Lys Leu Asn Tyr Lys Pro Pro Gln Lys Ser Leu Lys
 20 25 30

Glu Leu Gln Glu Met Asp Lys Asp Asp Glu Ser Leu Thr Lys Tyr Lys
 35 40 45
 Lys Thr Leu Leu Gly Asp Val Pro Val Val Ala Asp Pro Thr Val Pro
 50 55 60
 Asn Val Thr Val Thr Arg Leu Ser Leu Val Cys Asp Ser Ala Pro Gly
 65 70 75 80
 Pro Ile Thr Met Asp Leu Thr Gly Asp Leu Glu Ala Leu Lys Lys Asp
 85 90 95
 Thr Phe Val Leu Lys Glu Gly Ile Glu Tyr Arg Val Lys Ile Asn Phe
 100 105 110
 Lys Val Asn Lys Asp Ile Val Ser Gly Leu Lys Tyr Val Gln His Thr
 115 120 125
 Tyr Arg Thr Gly Met Arg Val Asp Lys Ala Thr Phe Met Val Gly Ser
 130 135 140
 Tyr Gly Pro Arg Pro Glu Glu Tyr Glu Phe Leu Thr Pro Val Glu Glu
 145 150 155 160
 Ala Pro Lys Gly Met Leu Ala Arg Gly Thr Tyr His Asn Lys Ser Phe
 165 170 175
 Phe Thr Asp Asp Asp Lys Lys Gln Asp His Leu Thr Thr Trp Glu Trp Asn Leu
 180 185 190
 Ala Ile Lys Lys Asp Trp Thr Glu Glx
 195 200

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 96 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: DNA (genomic)
 (iii) HYPOTHETICAL: NO
 (iii) ANTI-SENSE: NO
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Synthetic
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCGAGCTCGG TACCCGGCCG GGGATCCATC GAGGGTAGGC CTGAATTCAG TAAAACCCCTC 60

GATGGATCCT CTAGAGTCGA CCTGCAGGCA AGCTTG 96

- (2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 204 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: protein
 (iii) HYPOTHETICAL: NO
 (iii) ANTI-SENSE: NO
 (v) FRAGMENT TYPE: N-terminal
 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Bovine
 (F) TISSUE TYPE: Brain
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Ala Glu Gln Glu Pro Thr Ala Glu Gln Leu Ala Gln Ile Ala Ala
 1 5 10 15

Glu Asn Glu Glu Asp Glu His Ser Val Asn Tyr Lys Pro Pro Ala Gln
 20 25 30
 Lys Ser Ile Gln Glu Ile Gln Glu Leu Asp Lys Asp Asp Glu Ser Leu
 35 40 45
 Arg Lys Tyr Lys Glu Ala Leu Leu Gly Arg Val Ala Val Ser Ala Asp
 50 55 60
 Pro Asn Val Pro Asn Val Val Thr Arg Leu Thr Leu Val Cys Ser
 65 70 75 80
 Thr Ala Pro Gly Pro Leu Glu Leu Asp Leu Thr Gly Asp Leu Glu Ser
 85 90 95
 Phe Lys Lys Gln Ser Phe Val Leu Lys Glu Gly Val Glu Tyr Arg Ile
 100 105 110
 Lys Ile Ser Phe Arg Val Asn Arg Glu Ile Val Ser Gly Met Lys Tyr
 115 120 125
 Ile Gln His Thr Tyr Arg Lys Gly Val Lys Ile Asp Lys Thr Asp Tyr
 130 135 140
 Met Val Gly Ser Tyr Gly Pro Arg Ala Glu Glu Tyr Glu Phe Leu Thr
 145 150 155 160
 Pro Met Glu Glu Ala Pro Lys Gly Met Leu Ala Arg Gly Ser Tyr Asn
 165 170 175
 Ile Lys Ser Arg Phe Thr Asp Asp Asp Arg Thr Asp His Leu Ser Trp
 180 185 190
 Glu Trp Asn Leu Thr Ile Lys Lys Glu Trp Lys Asp
 195 200

We claim:

1. An isolated, purified D4 protein.
2. The protein of claim 1 further comprising a pharmaceutical carrier for administration to a patient.
3. The protein of claim 1 further comprising a solution of hematopoietic cells in vitro.
4. An isolated nucleic acid sequence encoding all or part of the D4 protein, at least fourteen to seventeen nucleotides in length.
5. The sequence of claim 4 further comprising a label.
6. The sequence of claim 4 wherein the sequence is of human origin.
7. The sequence of claim 4 wherein the sequence is of murine origin.
8. An antibody immunoreactive with the D4 protein.
9. The antibody of claim 8 labelled with a label.
10. A method for modulating proliferation and differentiation of cells comprising modulating the amount of D4 the cells are exposed to.
11. The method of claim 10 wherein the cells are hematopoietic cells.
12. The method of claim 10 wherein the cells are selected from the group consisting of brain, pre-adipocytes, muscle cells, lung cells, prostate cells, skin (melanocytes) cells, kidney cells, ovaries, bladder cells, fatty and neuronal tissue.
13. The method of claim 10 wherein D4 is inhibited from expression.
14. The method of claim 13 wherein expression is inhibited using antisense or ribozyme technology.
15. The method of claim 10 wherein exogenous D4 protein is administered to the cells.
16. The method of claim 15 wherein nucleic acid

encoding D4 is administered to the cells in a vector for expression of the protein in the cells.

17. The method of claim 10 wherein the cells are treated with an inhibitor of D4.

18. The method of claim 11 wherein the hematopoietic cells are from a patient with leukemia.

19. The method of claim 11 wherein the hematopoietic cells are for administration to a bone-marrow transplant patient.

20. A method for identifying defects in D4 expression in cells comprising assaying the cells or the genetic material from the cells with an isolated nucleic acid sequence encoding all or part of the D4 protein, at least fourteen to seventeen nucleotides in length.

21. A method for making a non-human animal model for D4 expression and function comprising
constructing a vector targeting the D4 gene,
introducing the vector into embryonic stem cells,

screening the embryonic stem cells for homologous recombination of the vector with the endogenous D4 gene and deletion of the endogenous D4 gene, and

generation of animals deficient in the D4 gene from the embryonic stem cells with homologous recombination of the D4 gene.

22. The method of claim 21 wherein the animals are rodents.

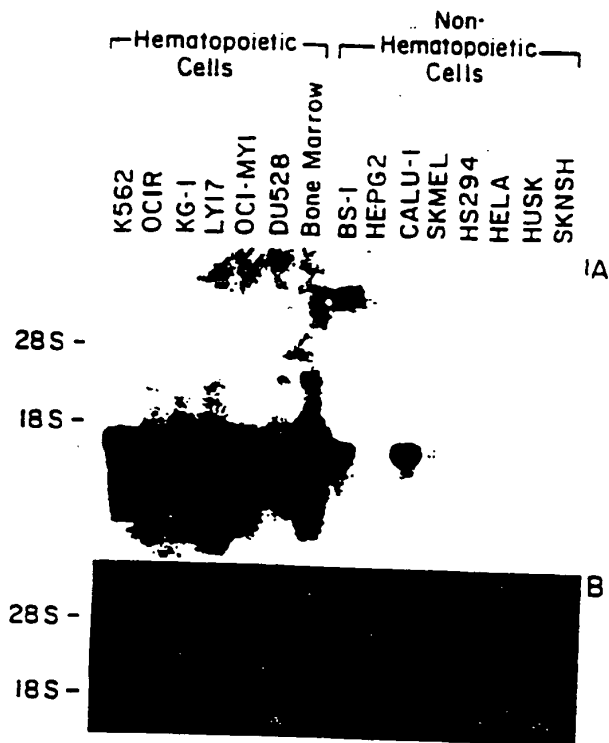
23. A non-human animal model for D4 expression and function formed by constructing a vector targeting the D4 gene,

introducing the vector into embryonic stem cells,

screening the embryonic stem cells for homologous recombination of the vector with the endogenous D4 gene and deletion of the endogenous D4

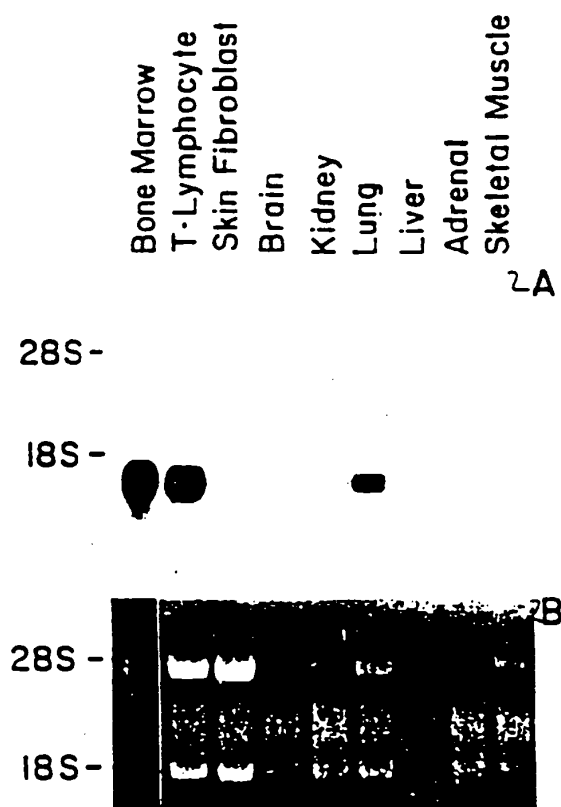
gene, and generation of animals deficient in the D4 gene from the embryonic stem cells with homologous recombination of the D4 gene.

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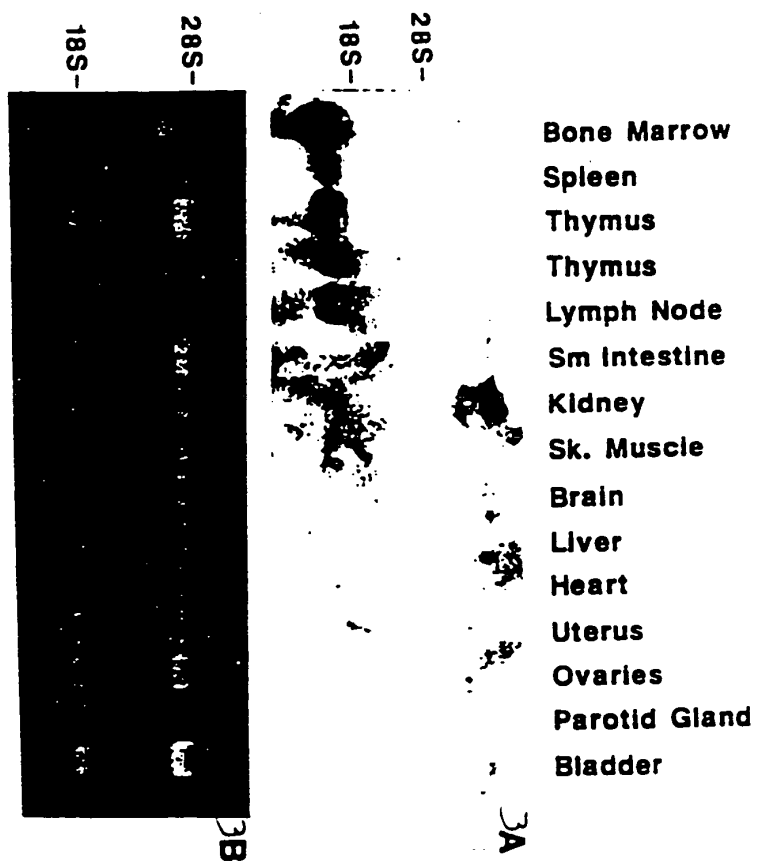
FIGURES 1A AND 1B

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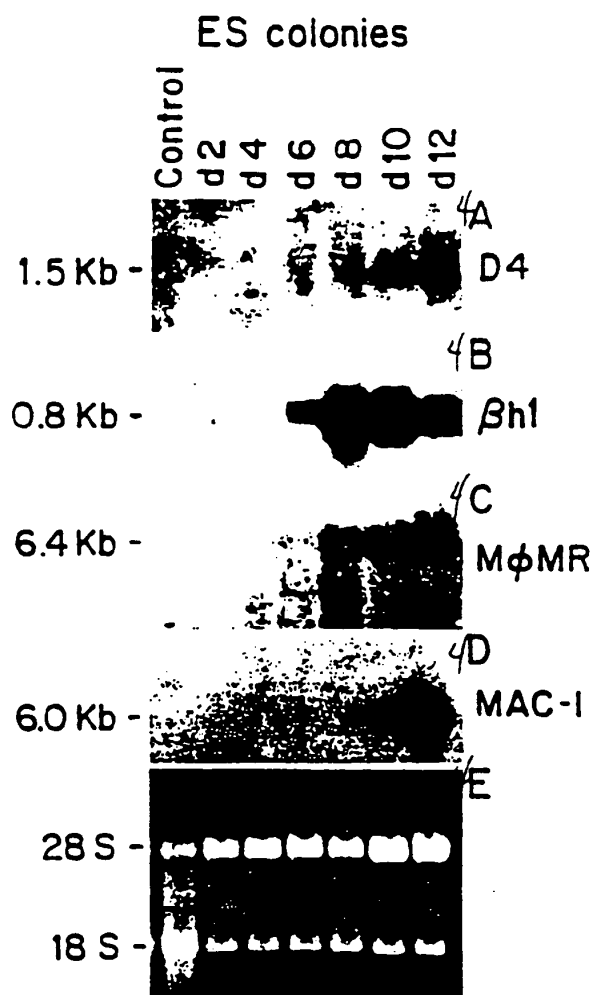


FIGURES 2A AND 2B

3 / 1 3

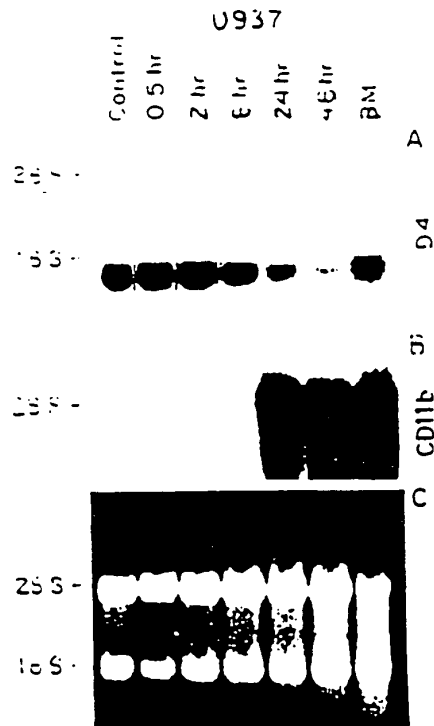


FIGURES 3 A and 3B

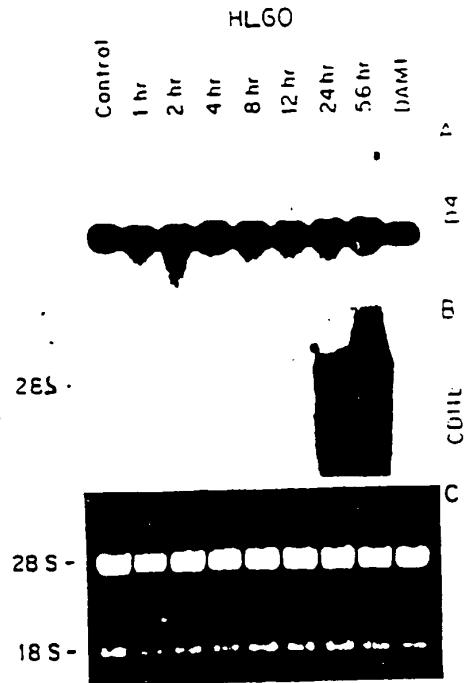


FIGURES 4A, B, C, D AND E

5a. U937 induced with TPA. The same blot was probed sequentially, (A) with D4 and (B) with CD11b.



5b. HL60 induced with DMSO.



5c. MEL induced with DMSO.

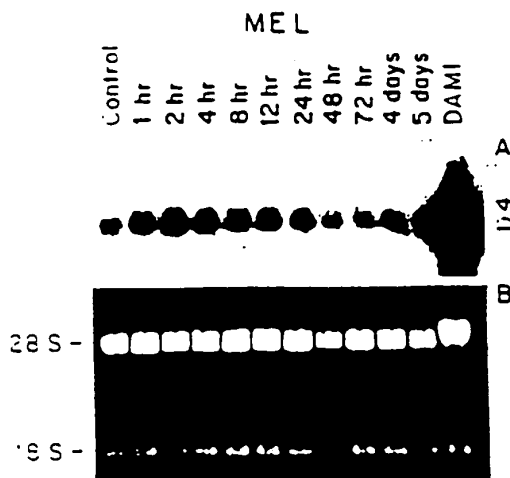


FIG 5A-C Northern analysis of hematopoietic cell lines at different times after initiation of induction.

BM: Normal bone marrow RNA
CD11B: Myeloid specific cell adhesion molecule () to show effective induction

U937: Myelomonocytic cell line

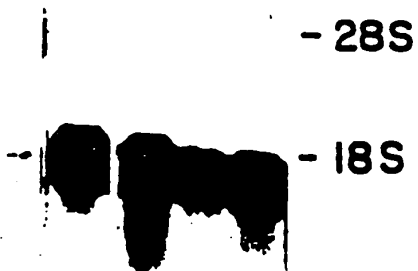
HL60: Myeloblast leukemia line

MEL: Murine erythroleukemia line

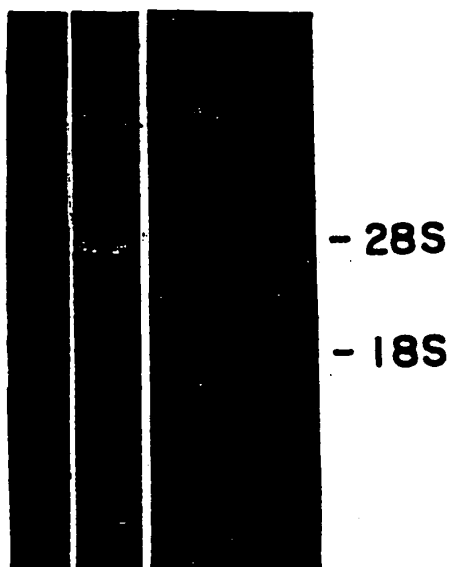
DAMI: Megakaryocytic line

FIGURE 6

7 A. Y.S. CELLS
 DAY 2.5 COLONIES
 DAY 4 COLONIES
 DAY 6 COLONIES (-SCF)
 DAY 6 COLONIES



7 B.



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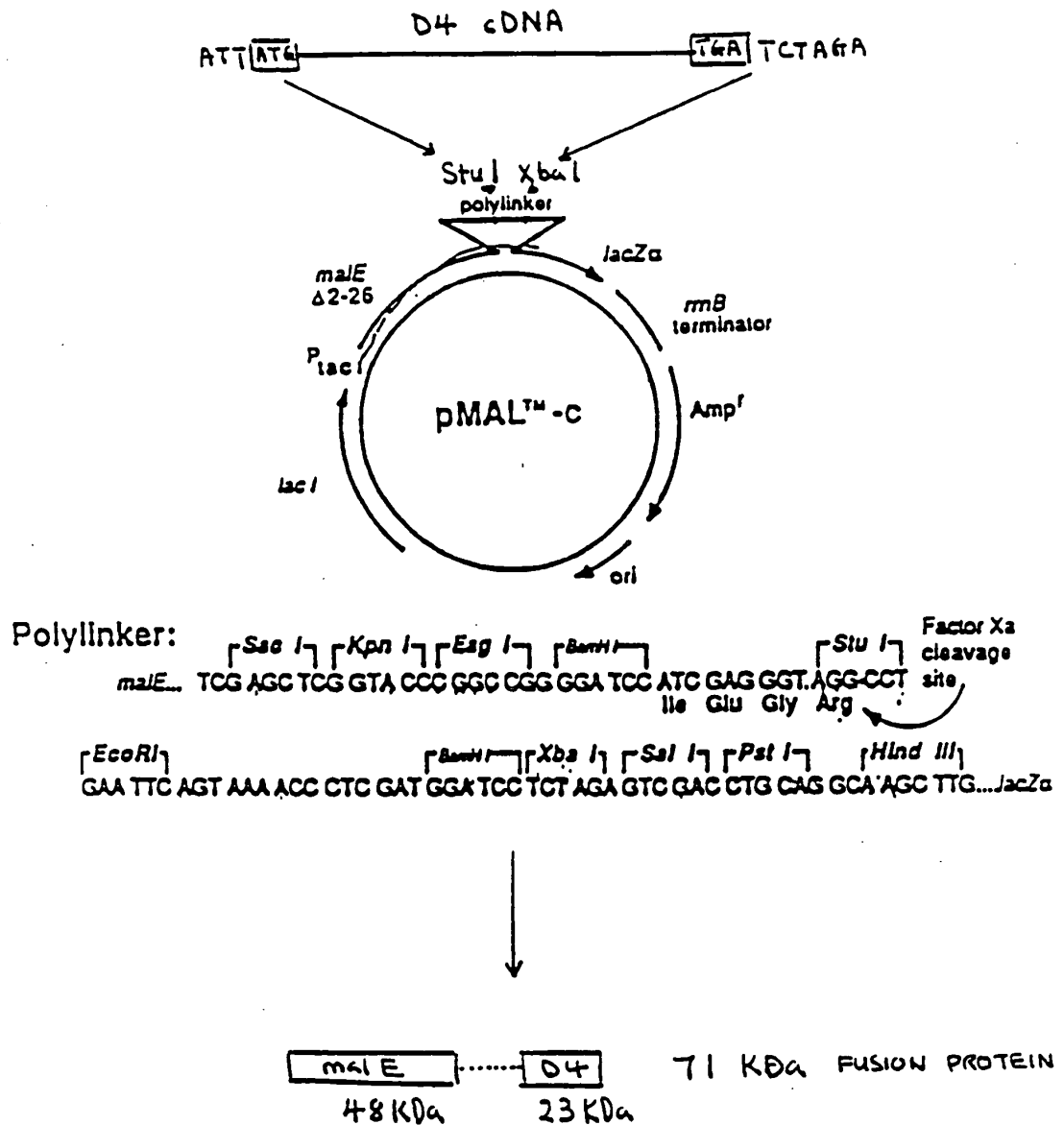
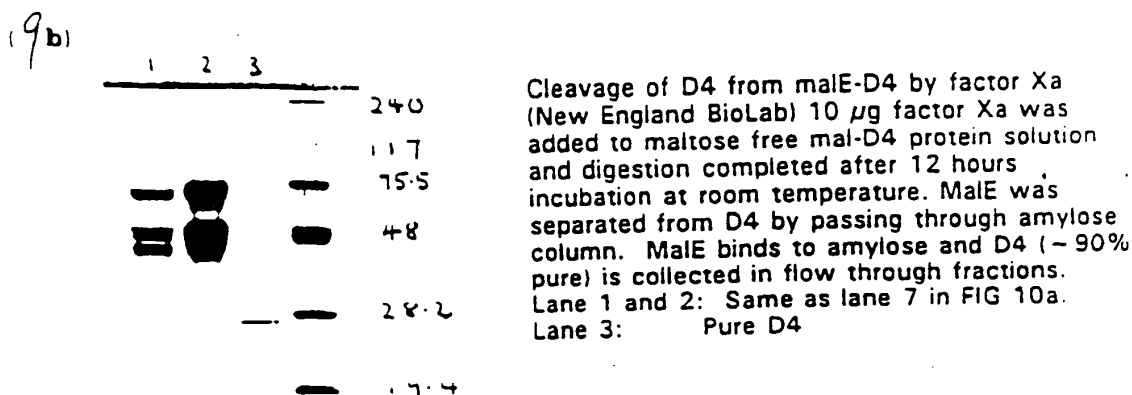
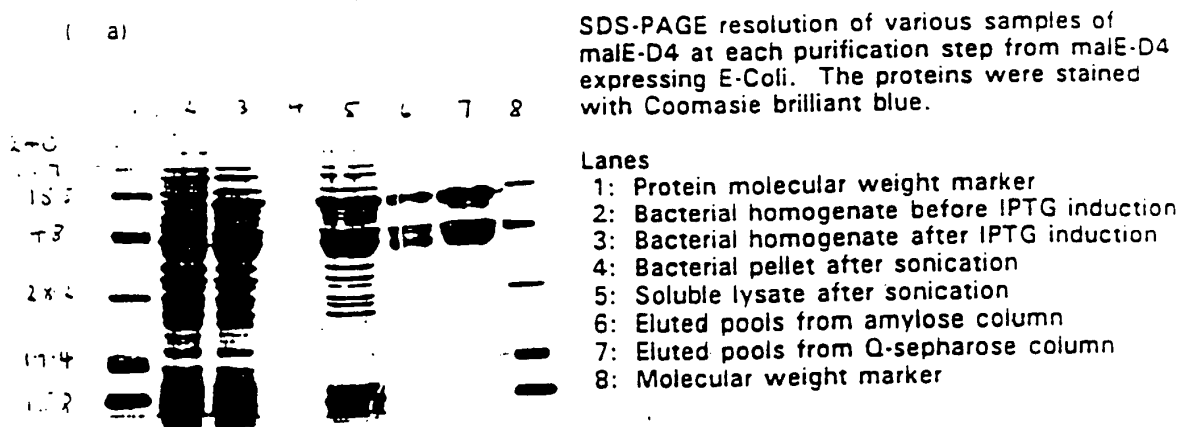
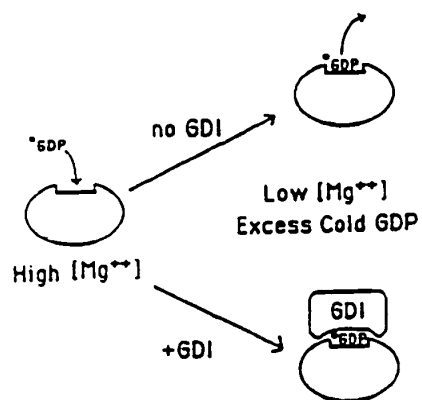


FIG 8 Construction of fusion-protein vector for expression of D4 in pMAL-c vector (New England BioLab).

FIG 9a Purification steps for deriving malE-D4 fusion and D4 protein





Principle of GDI-assay
FIG. 10A

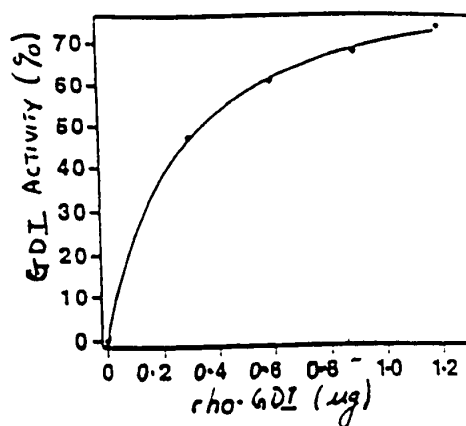


FIG 10B rhoGDI dose-response on
CDC42Hs

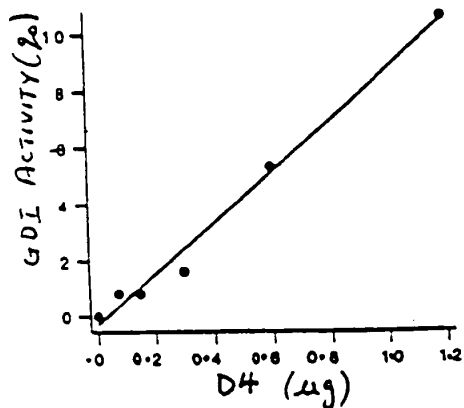


FIG 10C D4 dose-response on CDC42Hs

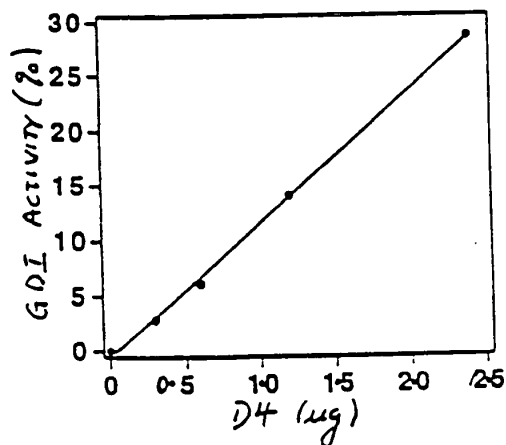


FIG 10D D4 dose-response on rac 1

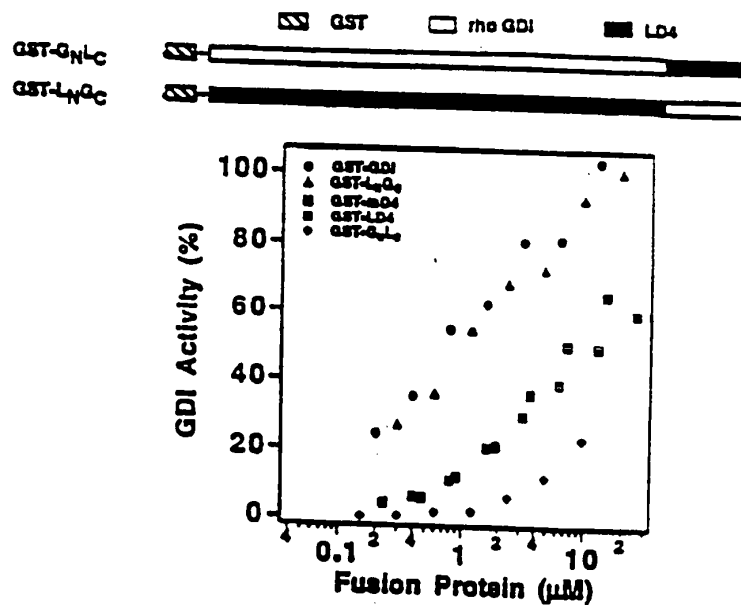
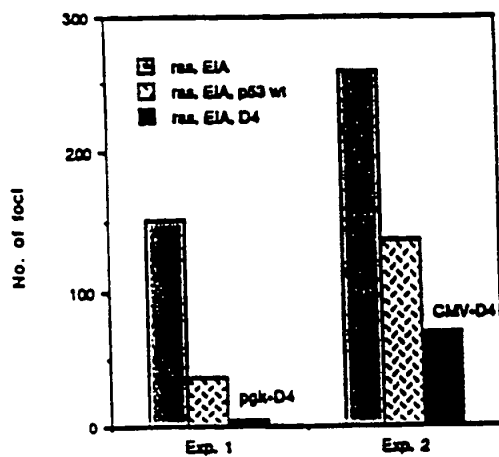


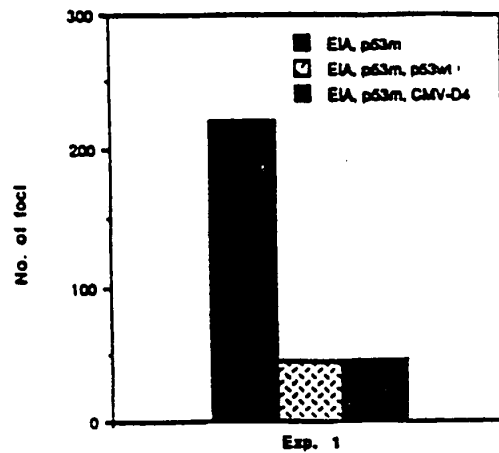
Figure 4

Fig 4 | Dose response profiles of GDI activity for chimeric proteins. Wild type and chimeric proteins of D4 (LD4) and rhoGDI were expressed as GST fusion proteins. GST-GDI = rhoGDI; GST-LD4 = human D4; GST-mD4 = murine D4; GST-L_NL_C = chimera of LD4-rhoGDI; GST-G_NL_C = chimera of rhoGDI-LD4. The chimeras involved switch of the carboxyl terminal 33 amino acids. The proteins were tested for their ability to inhibit the dissociation of [α -³²P]-GDP from Cdc42Hs. The activity curves show that the efficacy of the GDIs is dependent on the carboxyl terminal 33 amino acids. Note the replacement of LD4 C-terminal with that of rhoGDI shifts its activity curve of LD4 to superimpose on that of rhoGDI. Likewise, replacing rhoGDI C terminal with that of D4 shifts its GDI activity towards that of D4.

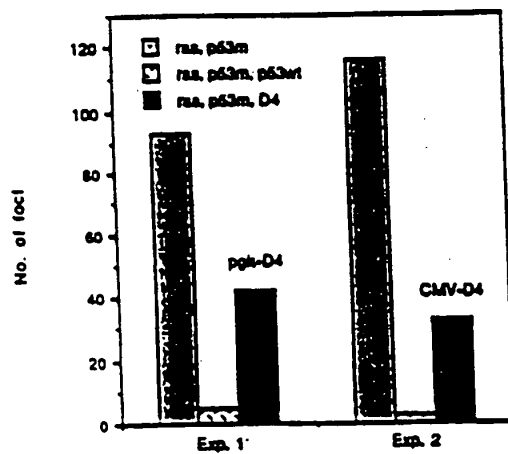
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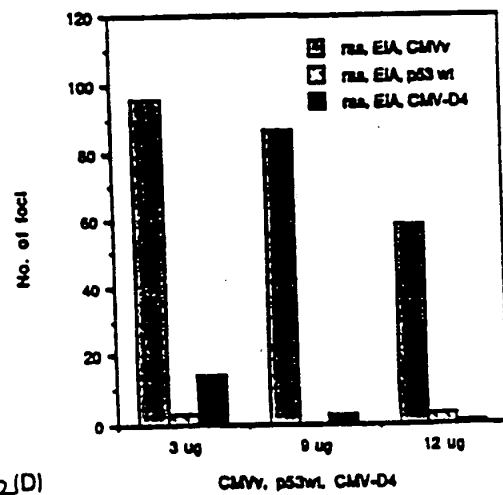
(12A)



(12B)



(12C)



(12D)

Figures 12A, B, C, AND D

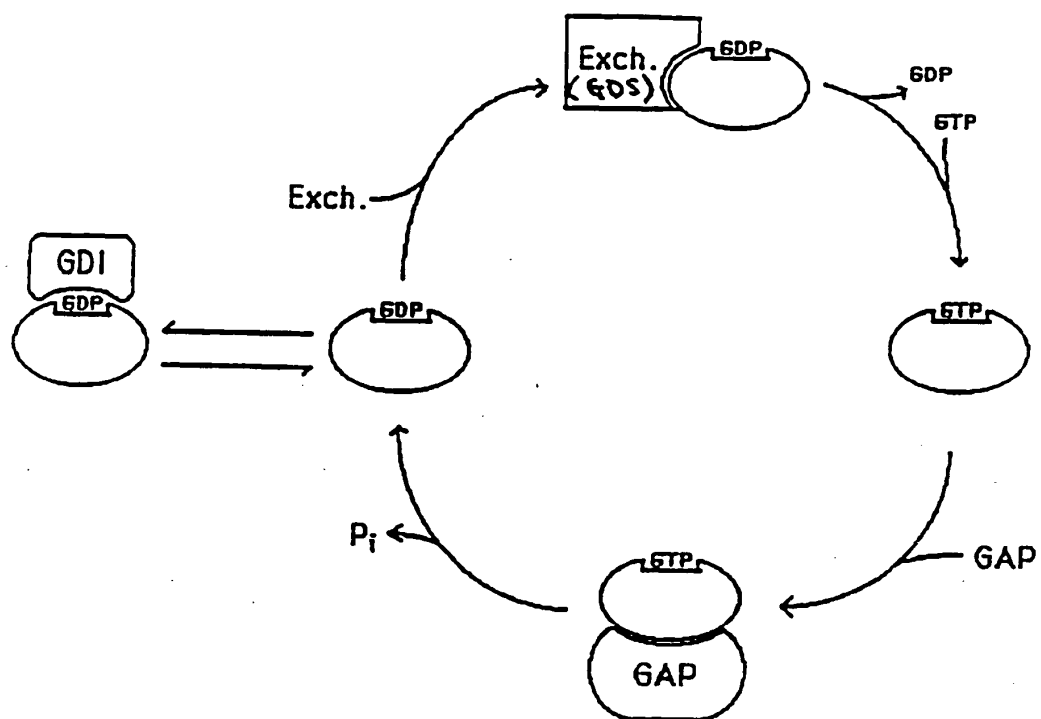


FIGURE 13

PCT/US 93/12074

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/12 C07K15/06 C12P21/08 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ONCOGENE vol. 5, 1990 pages 1321 - 1328 Y. FUKUMOTO ET AL.; 'Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the rho proteins, ras p21-like small GTP-binding proteins' cited in the application *abstract; Figures 1 and 7; discussion* --- -/--	1-5, 8-21, 23

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

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A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

24 March 1994

Date of mailing of the international search report

28. 04. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Yeats, S

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 168 , 1990 pages 1244 - 1252 H. FUJIOKA ET AL.; 'A small GTP-binding protein (G protein) recognized by smg p25A GDP-dissociating inhibitor (GDI) in human platelet membranes and GDI for this small G protein in human platelet cytosol' *summary; discussion*</p> <p>---</p>	4,6
A	<p>CURRENT OPINION IN CELL BIOLOGY vol. 3 , 1991 pages 185 - 191 T. EVANS ET AL.; 'The ras superfamilies: regulatory proteins and post-translational modifications' *page 187, last three lines - page 188, end of left-hand column*</p> <p>---</p>	1
P,X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF THE USA vol. 90 , 1993 pages 1479 - 1483 J.-M. LELIAS ET AL.; 'cDNA cloning of a human mRNA preferentially expressed in hematopoietic cells and with homology to a GDP-dissociation inhibitor for the rho GTP-binding proteins' *whole document*</p> <p>---</p>	1-6,8-20
P,X	<p>GENES, CHROMOSOMES AND CANCER vol. 8 , 1993 pages 253 - 261 C.N. ADRA ET AL.; 'Identification of a novel protein with GDP dissociation inhibitor activity for the Ras-like proteins CDC42Hs and Rac I' *whole document*</p> <p>-----</p>	1-5,7-23